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FASC I (APRIL 1961)

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FASC III (NOVEMBER 1961)

FASC. IV (DECEMBER 1961)

INDEX

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From the Department of Pharmacology (Professor Knud O Møller, M D) and the Department of Physical Chemistry (Professor C J Ballhausen, Ph D), University of Copenhagen, Denmark

Isolation of Hyaluronic Acid and Chondroitin-sulphuric Acid from Human Aortae

By

Sv. Bertelsen and K. Marcker

(Received September 1, 1960)

Much work has been devoted to the study of mucopolysaccharides in human and bovine aortic tissue BERTELSEN & JENSEN (1960) isolated both hyaluronic acid and chondroitin-sulphuric acid from human aortae and showed that the relative viscosity of the isolated crude mucopolysaccharide-mixtures decreased with age Comparing this finding with the results of a histochemical study on aortic tissue, the authors (BERTELSEN & JENSEN (1960)) assumed that the ratio chondroitin-sulphuric acid. hyaluronic acid increased with age In the biochemical investigation, however, the isolated substances were somewhat impure, which greatly hampered interpretation of the results The viscosity-determinations were carried out on impure mucopolysaccharide mixtures, because we feared that a too vigorous purification procedure would involve depolymerization of hyaluronic acid The isolated substances showed approximately the same degree of impurity (expressed as nitrogen percentage), which enabled us to compare their relative viscosities

The above-mentioned finding was in agreement with the work of FABER (1949), who arrived at the same conclusion by determining the sulphate content of human aortic tissue

KIRK & DYRBYE (1956), in a biochemical investigation, determined the acid hydrolyzable sulphate content in human aortae and found no variations in the sulphate content with age They expressed their results in terms of wet tissue weight

We hold that sulphate determinations performed direct on aortic tissue are not satisfactory For one thing, the amount of sulphate not bound by acid mucopolysaccharides is unknown Secondly, it is clear, especially

Aided by grant from the Danish State Science Foundation.

(BAN) = versene regular ® had been added up to a concentration of 0.01 M This was done in order to bind ferric ions, which are known to depolymerize hyaluronic acid The solution was then incubated with a crude commercial preparation of pancreatin (Pancreatin Solubile Novo) at a concentration of 50 mg per gram of tissue and at a temperature of 20°C for 2–3 days In order to precipitate products resulting from enzymic digestion of the protein, we added trichloroacetic acid up to a concentration of 15% After thorough cooling for one hour, the mixture was filtered several times The resulting solution was generally clear or nearly so, it was neutralized with a solution of potassium hydroxide, while being cooled and dialysed against running tap water (temperature 14°) for about 48 hours After the dialysis, the residual protein degradation products were removed by repeatedly shaking the solution with a mixture of chloroform and isoamyl alcohol (SEVAG (1934)) The solution was dialysed again for two days against running tap water and for two days against distilled water To the dialysate 2–3 volumes of cold absolute alcohol were added. The mixture was cooled at 4° for about 24 hours, and then centrifuged (3500 rpm) for 15 minutes [illegible]
in a vacuum de[illegible]
scopic and easily [illegible]

Paper Chromatography

The isolated substances were decomposed by hydrolysis with 2.0 N hydrochloric acid for 15 hours at 100° in sealed glass tubes Samples containing about 10 mg per 1 ml of acid were placed on Whatman filter papers No 1 in a volume of 20 µl and run in 2.6-lutidine/water (65 35) for 24 hours, using descending chromatography As reference substances were used authentic D glucosamine hydrochloride (Nutritional Biochemical Corporation) and D-galactosamine hydrochloride (Mann Research Laboratories) at concentrations of 10 mg per 1 ml of acid, 5 µl of each liquid were placed on the paper The chromatograms were developed with a 0.1 per cent solution of ninhydrin in acetone

Determination of the relative Content of Hyaluronic Acid and Chondroitin -sulphuric Acid

The method employed for determining the relative amounts of chondroitin sulphates and hyaluronic acid in the isolated substances was based on the substrate specificity of streptococcal hyaluronidase This is known to attack only the unsulphated mucopolysaccharides (MEYER & RAPPORT (1951)) By incubating the samples with this enzyme and dialysing the degradation products, it is possible to remove hyaluronic acid from a mixture of chondroitin sulphate and hyaluronic acid

Approximately 30 mg of the isolated substances were lyophilized and weighed The samples were then dissolved in McIlvaine standard buffer (pH-6,5), to which was added sodium chloride to a concentration of about 0.06 M, at a concentration of 10 mg per ml This solution was incubated with 50 µl streptococcal hyaluronidase*) for two days at 25–30° The incubated solution was carefully transferred to dialysis membranes and dialysed [illegible]

*) The bacterial enzyme was made by Dr V Faber of the Danish State Serum Institute, Copenhagen The enzyme was from a strain of a group of haemolytic streptococcus type 24 (A 24 N W) and contained 50 turbidity reducing units per ml of solution (FABER (1953))

from the work of MEYER *et al* (1957 & 1959) and BERENSON (1958), that the degree of sulphation in mucopolysaccharides containing sulphate may very well vary considerably This is especially true of heparin, but chondroitin sulphate A and chondroitin sulphate C have also shown varying degrees of sulphation

In the investigation reported here we have employed a method that we think should enable us to avoid the uncertainties of interpretation It consists in isolating the mucopolysaccharides in as pure a state as possible and in using the substrate specificity of bacterial hyaluronidase, which is known to attack only the unsulphated mucopolysaccharides, e g hyaluronic acid and chondroitin (MEYER & RAPPORT (1951))

This report also presents few results of hexosamine determinations on human aortic tissue

Materials and Methods.

After death (12–15 hours) 18 human aortae were obtained (from the Copenhagen County Hospital, Gentofte)

As far as possible, whole aortae from arcus to bifurcatio were employed, after careful removal of the adventitia by hand, the vessels were stored in acetone for about a month Only macroscopically normal aortic tissues were collected, if there was any question of elderly individuals with atherosclerotic aortae, the changed sections were discarded and only macroscopically normal parts were used Several tissue specimens from each purified vessel were microscopically examined, partly to ensure removal of adventitial tissue and partly to divide the vessels into groups The ages of the individuals ranged from 1 year to 80 years Owing to the small amount of tissue present in children, samples from individuals under 2 years of age were pooled

Partly by help of the microscopical examination, and partly by ordinary macroscopical examination, the material was divided according to calcareous content as well as to its macroscopic state Further aortae from a group of new-born children were included in the material Each of the samples in this group consisted of 12–15 aortae, and on each sample was performed a duplicate-test

Group 1 new born
Group 2 no medial calcium
Group 3 medial calcium, no atherosclerotic changes
Group 4 medial calcium, distinct atherosclerotic changes

For the microscopical picture of the aorta, see other publications (BERTELSEN (a in press) and BERTELSEN (b, in press))

The *method* used *for isolating purposes* is described below The acetone-dried and disintegrated aortic tissue was defatted with light petroleum and ether and then dried under reduced pressure and weighed The tissue was placed in a barbital buffer (pH – 7 8) to which tetracemin-tetranatrium (NFN)*) = tetrasodium edetate

*) Tetrasodium-ethylendiamino tetraacetic acid

(BAN) = versene regular ® had been added up to a concentration of 0.01 M This was done in order to bind ferric ions, which are known to depolymerize hyaluronic acid The solution was then incubated with a crude commercial preparation of pancreatin (Pancreatin Solubile Novo) at a concentration of 50 mg per gram of tissue and at a temperature of 20°C for 2–3 days In order to precipitate products resulting from enzymic digestion of the protein, we added trichloroacetic acid up to a concentration of 15% After thorough cooling for one hour, the mixture was filtered several times The resulting solution was generally clear or nearly so, it was neutralized with a solution of potassium hydroxide, while being cooled and dialysed against running tap water (temperature 14°) for about 48 hours After the dialysis, the residual protein degradation products were removed by repeatedly shaking the solution with a mixture of chloroform and isoamyl alcohol (SEVAG (1934)) The solution was dialysed again for two days against running tap water and for two days against distilled water To the dialysate 2–3 volumes of cold absolute alcohol were added The mixture was cooled at 4° for about 24 hours, and then centrifuged (3500 rpm) for 15 min [illegible]
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sulp[illegible]
with [illegible]
hya[illegible] chondroitin sulphate and hyaluronic acid

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*) The bacterial enzyme was made by Dr V Faber of the Danish State Serum Institute Copenhagen The enzyme was from a strain of a group of haemolytic streptococcus type 24 (A 24 N W) and contained 50 turbidity reducing units per ml of solution (FABER (1953))

Determination of Hexosamine Content of Aortic Tissue

Hexosamine content was determined on 20 human aortae thoracalis obtained fresh from autopsy at the Copenhagen County Hospital, Gentofte The age of the individuals ranged from 6 months to 83 years The determinations were made on tissue partly from macroscopically normal aortae and partly from macroscopically normal parts of atherosclerotic aortae

After careful removal of the adventitious tissue, as mentioned above, the freeze-dried and defatted intimal-media tissue was weighed on a torsion balance, cut into small pieces and placed in 5 ml glass ampoules with 4 ml of 2 0 N hydrochloric acid After sealing, the ampoules were left for hydrolysis for 15 hours at 100° on a glycerol bath The contents of the ampoules were filtered, and the hexosamine concentrations were determined in 3 ml of the filtrates by Blix's modification of the Elson & Morgan method (BLIX (1948))

Nitrogen and Sulphur Determinations

The nitrogen contents of the isolated mucopolysaccharides were determined by the Kjeldahl micro-analysis, about 10–20 mg of the substance being used for this purpose The sulphur content of the chondroitin sulphate fractions was determined by the method of (Zinneke (1951)) about 10 mg of the incubated and dialysed substance being employed

Results.

The purity of *the isolated substances* was checked by nitrogen determinations and paper chromatography The nitrogen content in the samples ranged from 3 7% to 4%, indicating a low percentage of protein impurities

The paper chromatography showed that hydrolysates of the isolated samples contained large amounts of galactosamine and glycosamine This method also showed that the samples had a high degree of purity, since only few and faintly coloured spots were visible in the chromatograms beside the strongly coloured spots, representing galactosamine and glycosamine

Hyaluronidase treatment confirmed that the isolated substance indeed consisted of mucopolysaccharides Streptococcal hyaluronidase and testicular hyaluronidase were added to solutions of the isolated substances, the effect of this addition being observed by viscometry, the characteristic degradation curves of hyaluronic acid and chondroitin-sulphuric acid were obtained

Table 1 shows the results obtained in determinations of the relative amounts of *hyaluronic acid and chondroitin-sulphuric acid* It is clearly seen that ratio chondroitin-sulphuric acid hyaluronic acid for the new-born is in the region of 1, increasing regularly with age The last column of the table gives the percentages of hyaluronic acid present in the isolated substances It is clear that the relative amount of hyaluronic acid in aortic tissue decreases with age

Table 1

Yield of mucopolysaccharides and percentage of hyaluronic acid in aortic tissue from individuals of various ages

Group	Age	Dry defatted tissue in g	Yield in mg	Yield as percentage of dry defatted tissue	Hyaluronic acid as percentage of acid mucopolysaccharides
1	0 year	1 89	28 32	1 5	59 50
	0 –	2 35	46 92	2 0	58 51
	0 –	2 41	40 90	1 7	65 61
2	*)1 2 years	2 35	35 21	1 5	48
	11 –	2 48	34 73	1 4	41
	14 –	3 01	42 20	1 4	43
	17	3 93	43 18	1 1	35
3	23 years	4 11	53 47	1 3	33
	24 –	3 99	39 86	1 0	10
	28 –	4 31	51 66	1 2	34
	31	4 06	60 93	1 5	31
	46 –	4 81	57 67	1 2	39
	61 –	5 32	53 17	1 0	10
4	62 years	5 91	65 00	1 1	10
	67 –	5 14	41 09	0 8	12
	70 –	7 25	36 26	0 5	15
	74	6 93	20 78	0 3	8
	76	7 24	28 94	0 4	19
	80 –	6 61	19 82	0 3	11

*) this sample consisted of 3 aortae.

It should further be noted that the yield of mucopolysaccharides decreases with age, a phenomenon also observed in previous investigations

Table 2 records the results of *hexosamine* determinations on dry defatted aortic tissue It is evident that the hexosamine content increases with age It should, however, be pointed out that the results have not been corrected for calcium, so that the content of hexosamine must be higher than appears from the table, especially in individuals over about 40 years of age

From the results in tables 1 and 2 it may therefore be safely concluded that the relative content of mucopolysaccharides in aortic tissue increases with age and that this increase is particularly attributable to an increase in the sulphated mucopolysaccharides

Sulphur analyses were made on some specimens from the chondroitin sulphate fractions The results are given in table 3 and show that the sulphur content is low and that specimens from elderly individuals contain less sulphur than those from younger persons

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Table 1

[illegible] of [illegible] acid in aortic [illegible]

Grc[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
1	0 year	1 89	28 32	1 5	59 50
	0 –	2.35	46 92	2.0	58 51
	0 –	2 41	40 90	1 7	65 61
2	*)1–2 years	2 35	35 21	1 5	48
	11 –	2 48	34 73	1 4	41
	14 –	3 01	42 20	1 4	43
	17 –	3 93	43 18	1 1	35
3	23 years	4 11	53 47	1 3	33
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	28 –	4 31	51 66	1 2	34
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	46	4 81	57 67	1 2	39
	61 –	5 32	53 17	1 0	10
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Table 2

Hexosamine content in aortic tissue from individuals of various ages (mg per 100 g dry, defatted tissue)

Age	Hexosamine content	gross appearance
6 months	900	natural
6 years	885	–
12 –	1036	–
23 –	1016	–
23 –	1038	–
25 –	957	–
28 –	943	–
31 –	1275	–
40 –	1197	–
40 –	1172	–
46 –	1099	–
53 –	1384	–
53 –	1099	slight atherosclerosis
65 –	1510	natural
65 –	1360	slight atherosclerosis
66 –	1340	– –
70 –	1145	pronounced atherosclerosis
70 –	1368	– –
76 –	1428	– –
83 –	1202	– –

Table 3

Sulphur content in the sulphate containing mucopolysaccharide fraction from aortic tissue in individuals of various ages

Age	Sulphur percentage
0 year	1 75
0 –	1 85
0 –	2 04
1–2 years	1 80
14 –	1 47
24 –	1 60
28 –	1 53
46 –	1 12
61 –	1 19
62 –	1 31
67 –	1 33
70 –	1 21

Discussion.

BERTELSEN & JENSEN (1960) *in a previous study found* that there was a decrease in the viscosity of the isolated mucopolysaccharides with age This decrease may be due either to the amount of hyaluronic acid de-

creasing in proportion to the amount of chondroitin sulphuric acid or to depolymerization of the hyaluronic acid molecule The investigation reported here makes it obvious that the relative amount of hyaluronic acid decreases markedly with age, although from the results obtained we cannot form any opinion of its molecular size and possible variation with age

The above-mentioned results are in accordance with the findings of MEYER *et al* (1957 & 1959), who demonstrated that the hyaluronic acid fraction in aortae from old persons was markedly decreased, if not absent

It should be stressed that our results show that hyaluronic acid is present in aortic tissue from all age groups, irrespective of the degree of atherosclerosis

The hexosamine determination clearly indicates an increase in the amount of acid mucopolysaccharides, whereas the yield of mucopolysaccharides decreases with age The decrease in the yield with age is in accordance with the finding of DYRBYE & KIRK (1957) The explanation of this inconsistency is not entirely clear, but it should be pointed out that the determinations were performed on dry, defatted, but calcareous tissue The decrease in the yield is, however, in our opinion greater than can be accounted for by correction for calcium content We must therefore assume that for some reason the acid mucopolysaccharides become more firmly bound to protein with age

As mentioned, KIRK & DYRBYE (1956) in their investigations found no significant variation in acid hydrolysable sulphate concentrations with age whereas M FABER (1949) in his sulphate determinations found that the "active part" of aortic tissue (dry weight corrected for lipoid and calcium) contained a percentage of sulphate rising with age The reason why Kirk & Dyrbye found no variation was no doubt that the determinations were carried out on wet, fat containing and calcareous tissue

KIRK & DYRBYE (1956) found further that the hexosamine content of aortic tissue was unchanged with age These results were expressed in proportion to wet, lipoid-containing calcareous tissue It is known that there is a vigorous increase in the amount of lipoid even from the age of 20–25 years, as well as in the amount of calcium in aorta (LANSING (1950 & 1952), BERTELSEN (1960)), and it therefore seems evident that the finding of a constant amount of hexosamine in material, without correcting for lipoid and calcium, may in fact mean a relative increase compared with dry, defatted and decalcified tissue Thus our results, which are for dry defatted tissue, indirectly confirm the results obtained by Kirk & Dyrbye

In hexosamine determinations on tissue, the amounts of hexosamine attached to acid mucopolysaccharides, as well as the amount attached

to mucoids and glycoproteins, are determined BERTELSEN (1960), in a previous investigation, has shown that the increase in the hexosamine content with advancing years is attributable to an increase in acid mucopolysaccharides, as well as neutral mucopolysaccharides and *glycoproteins*

Chondroitin-sulphuric acid – in combination with tissue protein – forms a gel with a large amount of free anionic groups In vitro experiments (BOYD *et al* (1951)) show that chondroitin-sulphuric acid may bind large amounts of calcium ions from solution GLIMCHER & *collaborators* (1957 & 1959) showed by in vitro experiments *that it is possible to produce* calcification of collagenous tissue by depolymerization of the ground substance and concluded that vascular chondroitin sulphate binds large amounts *of calcium ions, which are reliberated by depolymerization of* chondroitin sulphate

Several experiments (RUBIN *et al* (1951), ROBINSON (1952), ROUILLER *et al* (1952) and ROBINSON *et al* (1957)), during recent years seem to indicate that the presence of chondroitin sulphuric acid is necessary to initiate *mineralization and that precipitation of calcium salts as crystals* takes place in close relation to collagenous fibrils Besides chondroitin-sulphuric acid, fibrils and inorganic salts, the mineralization requires a number of unknown factors, *which may either take part in the calcification* process or merely act as catalysts for the process (SOBEL (1955))

BERTELSEN (a and b, in press) in histochemical investigations has demonstrated that mineralization of the vascular wall takes place in the ground substance, presumably in close relation to collagenous or collagen-like *fibrils This holds good of both the medial mineralization*, which takes place fairly early, and the sporadically occurring intimal mineralization, which is considerably less frequent

It would appear from table 3 that the sulphur content is very low There may be many explanations of this phenomenon, but the most obvious seems to be that the chondroitin sulphate fraction contains a polysaccharide with a low content of sulphur and that the amount of this polysaccharide increases slightly with age (table 3) According to MEYER *et al* (1959), heparitin sulphate contains maximally one sulphate group per disaccharide unit, whereas heparin monosulphate (isolated from ox lung by JORPES & GARDELL (1948)) has free amino groups, in contrast to heparitin sulphate, *in* which half of the NH_2-groups are acetylated, half sulphated It is probable that the low sulphur content is due to the presence of heparin monosulphate, heparitin sulphate or polysaccharides with *a very low degree of sulphation in the aortic* wall

Conclusion and Summary

It is shown that the hexosamine content in aortic tissue increases with age

The substrate specificity of bacterial hyaluronidase is used to determine the relative relationship between sulphated and non-sulphated mucopoly saccharides It is shown that the increase in acid mucopolysaccharides is due to an increase in chondroitin sulphates

From sulphur analyses made on the mucopolysaccharide fraction containing sulphate it is suggested that aortic tissue contains a polysac charide with a low degree of sulphation

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From the Pharmacological Laboratories of Dumex Ltd, (Formerly Medicinalco Ltd)
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Studies on Algesimetry by Electrical Stimulation of the Mouse Tail

By

Per Lund Nilsen

(Received October 17, 1960)

Existing experimental methods for testing analgesic activity can be divided into groups according to the stimuli used for producing pain The stimuli most frequently used are mechanical, thermal, electrical and chemical (GOETZL *et al* 1943; PFEIFFER *et al* 1948) All of them can be applied to mice and rats, which for many reasons are the animals most widely used in experimental algesimetry

In one of these methods, first devised by J F REINHARD and E J DE BEER, and later described and modified by BURN *et al* (1952), GREWAL (1952) and CUGURRA (1954), electrical stimuli are applied to the tails of mice When the animals respond by squeaking, this is regarded as a sign that the stimuli have been noci-ceptive

This method has been modified in our laboratories in several ways, the most important being that the normal pain threshold of each individual mouse is determined before the experiment The individual variation is extremely high, if it is not determined beforehand, the error in determining analgesic effect would be so great that minor differences would be concealed Establishment of the threshold increase for each individual mouse makes the method more accurate and more sensitive

Method.

Animals Male mice of a single strain weighing 15–20 g are used

Apparatus The mice are placed in cylindrical holders, with tops made of plexiglass, (for observing the animals under test) The back piece can be moved vertically and has a groove through which the tail can protrude

The electrodes are made of two cannulas no 20 attached to a piece of plastic-material 12 mm apart *They are inserted in the tail just beneath the skin,* the distal electrode being 5–6 mm from the tip of the tail

Stimuli of the square wave type are given by an impulse generator Intensity, duration and frequency can be varied independently In all the experiments described in this paper the stimuli lasted for 20 milliseconds and were given at intervals of 1 per second

The stimulus strength (voltage) is measured by a cathode ray oscillograph used as a peak voltmeter

Between the generator and the electrodes is a key by means of which the circuit can be opened and closed at will

Procedure Before the drug is given the pain threshold of each mouse is determined The voltage is increased stepwise by 100% from 2 to 64 volts until the animal responds by squeaking on the first or second shock. In this way the animals are divided into six groups according to their normal pain threshold

Squeaks alone are regarded as positive pain reactions, and animals failing to respond to four consecutive stimuli, when tested at the same voltage as for the pre-treatment threshold are defined as showing a "positive analgesic reaction"

All compounds were dissolved in physiological saline or suspended in 5% gum acacia and given intraperitoneally The analgesic effect was measured at intervals after injection, and the results are expressed as the percentage of animals showing a positive analgesic reaction, as defined above

Results.

A The Pain Threshold in normal, untreated Mice

Site of stimulation Different parts of the tail vary in sensitivity to electrical stimuli, as can be seen from table 1 A group of 87 mice were stimulated on three consecutive days at the tip, middle and base of the tail, and the number of animals squeaking at the first or second shock was determined The sensitivity increases from the tip to the base of the tail

For practical reasons we have used the tip of the tail as site of stimulation in all our experiments

Table I

Influence of site of stimulation on pain response
Male mice 20-25 g Electrical stimuli 16 volts.

Stimuli applied	No of mice	No of mice squeaking		P (%)*)
Tip	87	52	(60%)	99–99 9
Middle	87	72	(83%)	
Base	87	86	(99%)	99 9

*) calculated from the χ^2 test as described by Burn et al (1952)

Table 2

Relationship between sensitivity to electrical stimuli and weight (age) of male mice Stimulus intensity 16 volts

Weight (grams)	No of mice	No of mice squeaking		P (%)
10–20	95	73	(77%)	98–99
20–30	49	27	(55%)	

The effect of age The sensitivity decreases with increasing body-weight This means that it also decreases with increasing age (table 2)

The effect of sex There is a small, though significant, difference in pain sensitivity between the sexes when constant stimuli are applied to animals of the same strain and age The males are more sensitive than the females (table 3)

Strain The sensitivity and frequency distribution of the normal pain threshold in two different strains of mice have been investigated on males of the same age and weight

As can be seen from fig 1, there is a considerable difference in sensitivity to electrical stimuli between the two strains of mice

The reproducibility of normal pain threshold A group of 100 male mice of strain I was tested once or several times daily for 4 days The results are given in table 4

There is a significant increase in the number of animals responding between the first and the second test of the day, even when the interval between readings is 3 to 4 hours

When the animals are stimulated more than twice on the same day, the pain threshold of the group remains constant and equal to that at the second determination

The day-to-day variation in the group's normal pain threshold is small and without significance

Table 3

Influence of sex on pain response Mice of strain I 10–20 g Electrical stimuli 16 volts

Sex	No of mice	No of mice squeaking		P (%)
F	295	192	(65%)	99–99 9
M	1308	966	(74%)	

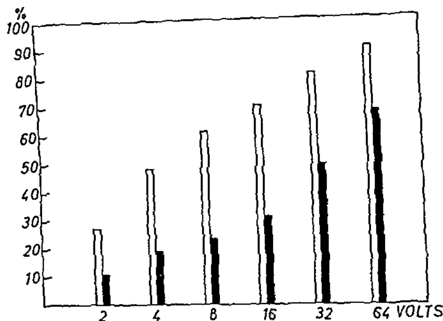


Fig 1 No of mice squeaking, with various intensities of stimuli (2–64 volts)
Male mice, 15–20 g
□ Strain I [illegible]
■

Table 4

Reproducibility of normal pain threshold Male mice of strain I, 15–20 g
Electrical stimuli 8 volts

Date	Stimulation number	Time	No of mice	No of mice squeaking	P (%)
13/11 58	1	10 30	100	57	98–99
–	2	13 30	100	74	
14/11 58	1	09 30	100	61	95–98
–	2	13 30	100	75	
17/11 58	1	09 30	100	59	
–	2	13 05	100	70	80–90
–	3	14 05	100	75	30–80
–	4	15 05	100	73	10–20
24/11-58	1	09 30	100	61	

In consequence of these investigations we have determined the normal pain thresholds in two tests at least, only the final reading being used

Tissue damage We have applied stimuli of maximum intensity to 100 male mice, 20–25 g, on three consecutive days The percentage of squeaking animals was 77, 77, and 71 Thus there was no systematic variation

B The Pain Threshold after Administration of Analgesics

The influence of the normal threshold Frequency distribution under analgesics The relationship between normal pain threshold and analgesic response has been determined in tests on 872 mice of strain I and 222 mice of strain II, all males weighing 15–20 g The animals were given 12 5 mg codeine phosphate per kg bodyweight intraperitoneally, and the pain threshold was determined 15 min after the injection

As can be seen from tables 5a and 5b, there is a correlation between the normal pain threshold and the corresponding value after an injection of codeine phosphate Animals with the lowest initial threshold are the most sensitive to the effect of codeine

Strain and age Analgesic response, i e the percentage of animals showing analgesia, varies according to the strain and age, as is shown in tables 5 and 6

Table 5 shows that, when given 12 5 mg codeine phosphate per kg bodyweight intraperitoneally, 53% of the animals of strain I exhibit analgesia, whereas the corresponding value for mice of strain II is only 38%

Table 5 a

Relationship between normal pain threshold and analgesic response determ ned 15 min after intraperitoneal injection of 12 5 mg codeine phosphate per kg bodyweight Male mice, 15 20 g strain I & II

Normal pain threshold (volts)	Strain I			Strain II		
	No of mice	No of mice showing analgesia		No of mice	No of mice showing analgesia	
2	242	150	(62%)	25	17	(68%)
4	156	93	(60%)	28	15	(54%)
8	92	49	(53%)	11	4	(36%)
16	108	71	(66%)	40	18	(45%)
32	147	56	(38%)	57	16	(28%)
64	127	43	(34%)	61	15	(25%)
Total Average	872	462	(53%)	222	85	(38%)

Table 5 b

Normal pain threshold (volts)	Strain	No. of mice	No. of mice showing analgesia		P (%)
<8	I	490	293	(60%)	99.9
>8		382	170	(45%)	
<8	II	64	36	(56%)	99.9
>8		158	49	(31%)	

This may be mainly due to the fact that relatively more animals having a high normal pain threshold and a correspondingly low sensitivity to analgaesics were found in strain II. However, it seems as if even the animals of this strain that have the same threshold as animals of strain I were slightly less sensitive.

Table 6. 560 male mice of strain II were divided into groups, according to bodyweight, (i.e. age), 12.5 mg codeine phosphate per kg was given intraperitoneally, and the number of mice showing analgesia was determined 15 min. after injection. The table suggests a slight decrease in sensitivity to codeine phosphate with increasing bodyweight, but the difference between the two groups was not statistically significant.

Sex. No significant difference in the response to analgesics was observed between the sexes when animals of the same strain and bodyweight were used. In a special series of experiments 124 out of 276 (i.e. 45%) female mice of strain I, 15–20 g, showed analgesia 15 min. after an intraperitoneal injection of 12.5 mg codeine phosphate per kg.

Table 6

Effect of weight (age) on the analgesic response, determined 15 min. after intraperitoneal injection of 12.5 mg codeine phosphate per kg body weight
Male mice, strain II

Weight (grams)	No. of mice	No. of mice showing analgesia		P (%)
10–20	233	88	(38%)	80–90
20–30	327	103	(32%)	

Table 7.

Analgesic potency of some derivatives of salicylic acid
Male mice of strain I, 15–20 g

Compound	Dose mg/kg	No of mice	No of mice showing analgesia	ED 50 mg/kg	Slope of regression line (see text)
Acetylsalicylic acid	250 500	92 43	23 (25%) 18 (42%)	580 (410–1000)	2 21
Salicylamide	125 250 500	10 10 8	1 (10%) 7 (70%) 8 (100%)	216 (122–357)	6 92
Phenazone salicylate	200 400 500	20 20 8	8 (40%) 18 (90%) 8 (100%)	207 (187–378)	5 63

body weight This figure is in complete agreement with that found for male mice, as can be seen from table 10

In order to test the reliability of the method, the analgesic effect of some antipyretic as well as some morphinomimetic drugs was investigated The results are given in tables 7–10 Among the antipyretics, the pyrazolone derivatives – in particular amidopyrine – had the greatest analgesic effect Less active were the derivatives of salicylic acid and the aniline compounds, phenacetin and acetanilide

The results have been analysed statistically by the methods described by BURN *et al* (1952) From the percentage of animals showing analgesia probits were calculated and plotted against log-dose, as shown in the

Table 8

Analgesic potency of aniline derivatives
Male mice of strain I, 15–20 g

Compound	Dose mg/kg	No of mice	No of mice showing analgesia	ED 50 mg/kg	Slope
Phenacetin	100 200 300 500	30 143 20 29	7 (23%) 64 (45%) 13 (65%) 21 (72%)	270 (210–420)	1 87
Acetanilide	200 400	32 17	13 (41%) 15 (88%)	225 (200–320)	4 70

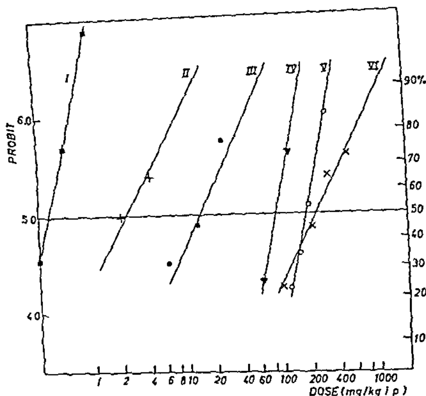


Fig 2 The dose response ...

V Phenazone
VI Phenacetin
Abscissa Log dose (mg per kg bodyweight)
Ordinate Percentage of mice showing analgesia

examples in fig 2 The observations were then corrected for their weights, and the regression lines of doses on weighed probits calculated as described by BURN *et al* (1952) The slopes of the regression lines and the median effective doses are given in the tables 7–10 In some instances the fiducial limits were also calculated By means of the χ^2 test all the regression lines were tested for rectilinearity, as described by BURN *et al* (1952)

The slopes of the curves for codeine, pethidine and morphine did not differ significantly from one another ($\chi^2 = 5\ 13$ with 4 degrees of freedom) The common slope of the regression lines for these three compounds is 1 62

Table 7

Analgesic potency of some derivatives of salicylic acid
Male mice of strain I, 15–20 g

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Acetanilide	200 400	32 17	13 (41%) 15 (88%)	225 (200–320)	4 70

Table 10

Analgesic potency of some morphinomimetic substances
Male mice of strain I 15–20 g

Compound	Dose mg/kg	No of mice	No of mice showing analgesia	ED 50 mg/kg	Slope
Codeine phosphate	6 25 12 5 25	70 1267 35	22 (31%) 570 (45%) 27 (77%)	14 7 (13 3–18 2)	1 78
Morphine hydrochloride	2 4	42 48	20 (48%) 31 (65%)	2.11	1 46
Pethidine hydrochloride	3 6 12	50 45 45	10 (20%) 19 (42%) 24 (53%)	9 48 (7 01–18 6)	1 52
Dextromoramide bitartrate	0 25 0 5 1	33 33 33	11 (33%) 25 (76%) 32 (97%)	0 33 (0 42–0 27)	3 9

The most active of the morphinomimetic drugs is dextromoramide (Palfium ®), which in this test had an analgesic effect approximately seven times that of morphine Codeine was, as expected, the least active compound of the group

Discussion

A method for testing analgesic compounds has been applied to various well known substances, and it has been demonstrated that a rectilinear relationship exists between the logarithm of the doses of analgesics and the probit of the number of animals showing analgesia This is true for both antipyretic and morphinomimetic analgesics

Most authors have been able to measure the analgesic action of the morphinomimetic compounds, such as morphine itself, methadone, pethidine etc , in non toxic doses, but this does not seem to be possible with the antipyretic analgesics Some authors (GREWAL 1952, SMITH *et al* 1943, WOOLFE & MACDONALD 1944) have been able to demonstrate slight analgesic activity of these compounds, but they could not give dose response curves because the doses needed evoked toxic symptoms, and sometimes were even lethal

Table 9

Analgesic potency of some pyrazolone derivatives
Male mice of strain I, 15–20 g

Compound	Dose mg/kg	No of mice	No of mice showing analgesia	ED 50 mg/kg	Slope
Phenazone	62 5	20	0 (0%)		
	125	67	14 (21%)		
	150	17	7 (41%)		
	188	144	76 (53%)	197	4 41
	300	182	151 (83%)	(180–216)	
Isopropylphenazone	100	50	10 (20%)		
	150	58	25 (43%)	141	3 70
	200	50	33 (66%)	(127–158)	
Isobutylphenazone	100	10	3 (30%)	150*)	
	200	10	10 (100%)	(approx)	
Amidopyrine	62 5	63	16 (25%)		
	125	51	37 (73%)	89	4 50
	250	15	15 (100%)	(76–105)	
Phenylbutazone	31 5	27	0 (0%)		
	62 5	28	2 (7%)		
	125	27	5 (19%)	174	3 56
	250	28	21 (75%)	(161–192)	

*) calculated from the common slope for the regression line of salicylamide etc (b = 4 59)

The antipyretic analgesics, with exception of phenacetin (and perhaps also acetylsalicylic acid), had a much steeper slope, which may be identical with that of salicylamide, phenazone salicylate, acetanilide, phenazone, isopropylphenazone, amidopyrine and phenylbutazone ($\chi^2 = 12\ 62$, 14 degrees of freedom) The common slope for the regression line of these compounds is 4 59

Acetylsalicylic acid and phenacetin have a less steep slope (b = 1 87), differing significantly from the common slope of the other antipyretic analgesics mentioned above

In calculating the median effective doses and the fiducial limits, the common slopes used were b = 4 59 for the antipyretic analgesics (except phenacetin and acetylsalicylic acid), b = 1 87 for phenacetin and acetylsalicylic acid and b = 1 62 for codeine, pethidine and morphine For dextromoramide the slope found was b = 3 9, and this has been used in the calculations

in non-toxic doses The ED 50's of the compounds mentioned were found to be 160, 165, 260 and 98 mg/kg bodyweight, respectively Compared with our results, it will be seen that acetylsalicylic acid was more active in this test, whereas the analgesic effects of phenacetin and amidopyrine were almost the same as those found by us

It has been pointed out here that the effect on mice of different analgesic compounds depends on the animal's normal pain threshold, which varies with strain and age For the evaluation of dose response curves and for comparing median effective dose of different drugs, it is therefore necessary to determine the pre-treatment threshold of the animals and divide them into groups accordingly Equal numbers from each of these groups should be used for each compound investigated

For the evaluating of weak analgesics it is advisable to use young mice of a sensitive strain

The analgesic activity of a new compound should be determined by comparing its effect with that of a well-known analgesic (e g codeine) determined on the same day and on the same type of animal

Summary.

A method for testing analgesic compounds in mice is described It is based on an all or-none response towards electrical stimuli of constant duration and frequency, but of varying intensity The pain threshold of each mouse is determined before and after the administration of the analgesic compounds, and the animals are defined as showing a "positive analgesic reaction" if the second pain threshold is found to be increased

The method has been applied to various wellknown analgesics, and it has been shown that the percentage of mice showing analgesia rises with increasing doses The ED 50 (mg/kg i p) has been determined for the antipyretic and morphinomimetic drugs, acetylsalicylic acid (580), [illegible]

Acknowledgement

I am grateful to Erik Jacobsen, M D for the statistical analysis of the results

My thanks are given to Mrs Kirsten Lovgreen and Miss Mona Rimestad for careful technical assistance

Exceptions are techniques described by KRAUSHAAR (1953) and WAY *et al.* (1953). KRAUSHAAR applied radiant heat or electrical stimuli to the tails of mice; WAY *et al.* applied graded pressure stimuli to the tails of rats and determined the grams of force necessary to elicit the squeak response.

SIEGMUND *et al.* (1957) produced "writhing" in mice by intraperitoneal injections of 2-phenyl-1,4-benzoquinone. The "writhing" syndrome is prevented by oral administration of acetylsalicylic acid, phenacetin and amidopyrine, given in graded non-toxic doses.

The methods of KRAUSHAAR and WAY differ from each other in the nature of the painful stimuli and the routes of administration, but they have two important points in common. The pre-treatment threshold of the animals was determined and only animals with high sensitivity were used. Moreover, the dose-response curves were based on all-or-none responses. The method described in this paper differs from theirs on one important point, namely that all animals were used, even those of low sensitivity.

The use of mice has several advantages. It permits selection of a uniform and sensitive strain of animals, which minimizes individual variation. It also permits, as shown, the evaluation of weak analgesics. Moreover, it is possible to use a large number of animals, which is essential for reasonable accurate results.

The use of electrical stimulation permits independent variation of intensity, duration and frequency of the stimuli. All of them can be measured in well defined units, and with square-waves the peak of intensity is reached instantaneously. Electrical stimuli of a reasonable intensity do not produce tissue damage, and repeated application of stimuli is therefore possible without loss of uniformity in response. A squeak as a response involves the participation of higher centres in the central nervous system, and for this reason it is preferable to other reflex mechanisms, for example "tail-flick", which can be provoked even by direct muscle stimulation.

The relative potency of the different morphinomimetic analgesic compounds investigated compares well with that found by most authors using other animal tests, (CAHEN *et al.* 1948, FRIEBEL & REICHLE 1956, CHRISTENSEN & TYE 1951, HAAS *et al.* 1953). The findings also conform with the relative effective doses used in the clinic, as compiled by EDDY *et al.* (1956).

Judged by the information in the literature, the "writhing" method applied to mice seems very sensitive. Thus SIEGMUND *et al.* (1957) were able to determine the median effective doses of sodium acetylsalicylate, acetylsalicylic acid, phenacetin and amidopyrine after oral administration

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From the Biological Department of Ferrosan Ltd
(Alf Lund, Ph D)

Comparative Studies of the Diuretics Acetazolamide, Aminometradine, Chlorothiazide and Theophylline

By

Ole E. Nielsen

(Received October 25, 1960)

The object of the study reported here has been to compare the action of theophylline, the classical diuretic, with those of modern mercury-free diuretics, in order to obtain a basis for evaluating the advantages, if any, possessed by the latter (aminometradine[1]), acetazolamide[2]), and chlorothiazide[3])) over this familiar predecessor

The comparison was made with special reference to the influences of the drugs on the excretions of water and electrolytes, as well as their actions on acid-base balance and glomerular filtration in normal animals and humans

About the mechanisms of action of these diuretics we are in the main well informed, that of theophylline having been analysed by MOLLER (1926) that of chlorothiazide by BEYER (1958), that of acetazolamide by MAREN *et al* (1954) and that of aminometradine by FORD *et al* (1957)

With our object in view, short term clearance experiments were made on rabbits, as well as a comparison of the effects of the drugs on rats Finally we aimed at throwing some light on the renal actions of the drugs in human subjects and on their duration

Experiments on Rabbits.

Experimental

Male rabbits were used weighing from 2 5 to 3 5 kg. The animals had had no access to food, but were allowed water *ad lib* before the experiment They were anaesthetised with urethane and pentobarbital Before the experiment was begun, a tracheal cannula

[1]) Mictine ®
[2]) Diamox ®
[3]) Salisan ®

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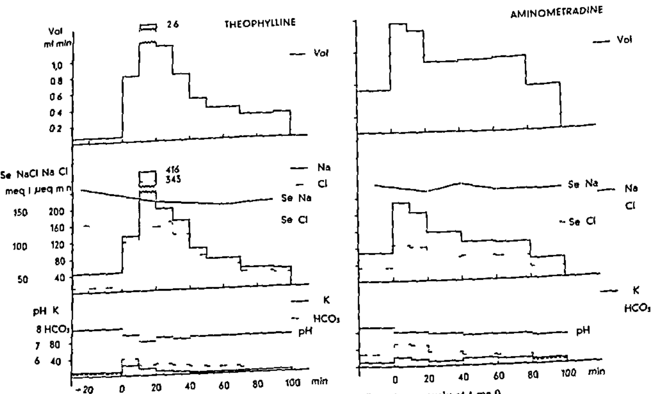


Fig 1 Rabbit no 276 Theophylline 20 mg/kg intravenously at t me 0
Rabb t no 246 Aminometradine 20 mg/kg intravenously at time 0

was inserted, to ensure a free air passage, and a glass cannula was introduced into the exteriorised bladder, to reduce the dead space between the kidneys and the cannula Blood samples were withdrawn through a cannula in the common carotid During the experiment physiological saline (0 33 ml/min) containing creatinine (1 mg/min) was infused continuously into an ear vein At the beginning of the experiment a large dose of creatinine (200 mg) was given intravenously Urine was collected for periods of 10 to 20 minutes After a suitable number of pre-periods, the diuretic was injected intravenously

Blank tests showed that the 4–6 ml of physiological saline in which the injected diuretics had been dissolved had no influence on the course of the diuresis or on electrolyte excretion

Analytical Methods.

Sodium and potassium were determined by flame photometry Chloride was determined titrimetrically (SCHALES & SCHALES 1941) Bicarbonate was determined manometrically by VAN SLYKE's macromethod (1947) Creatinine was measured spectrophotometrically after picric acid coupling in alkaline fluid Ammonia was determined by CONWAY's diffusion method (1947)

Theophylline

Three experiments were carried out, one with 10 and two with 20 mg theophylline per kg intravenously The effects on water and electrolyte excretions were closely similar in the three experiments Hence the results of one only will be given in detail (fig 1)

The urine volume discharged rose instantaneously and most markedly, but the rise was of short duration

Electrolyte excretion was dominated completely by the excretions of sodium and chloride, which increased almost as much as the urine volume Potassium and bicarbonate excretions were little affected when allowance is made for the pronounced diuresis

The increasing urine flow was accompanied by a slight fall in pH, as is also usually seen in simple water diuresis We can only conclude from these facts that theophylline has no great influence on acid-base excretion

In the experiment illustrated here a steady fall was seen in the serum sodium and serum chloride levels during the experimental period However, immediately after the last urine specimen had been withdrawn a tendency towards a rise was noticed, presumably a sign of dehydration

Chlorothiazide

Three experiments were done by intravenous injection of chlorothiazide (fig 2) The action of this drug was slower than that of theophylline, but of longer duration

The excretion of sodium likewise rose steadily and fell parallel with decrease in urine flow Chloride excretion increased less On the other hand, bicarbonate excretion increased so much as to compensate for the "chloride deficit" This is explained by the carbonic anhydrase inhibitory action of chlorothiazide That the amount of potassium excreted was somewhat larger than after injection of theophylline (measured in mEq/ml) was presumably due to the same mechanism of action, since the depressed hydrogen ion exchanged with sodium at re absorption was to some extent replaced by the potassium ion

The mild carbonic anhydrase inhibitory action was also reflected in the pH values measured, which displayed a slight tendency to rise, despite the distinctly alkaline urine of the pre-period

No unequivocal alteration was seen in serum electrolytes

Acetazolamide

Three experiments were done, one with 10 and two with 20 mg/kg, intravenously The excretion patterns in the three experiments were similar Fig 2 illustrates one of the experiments with 20 mg/kg

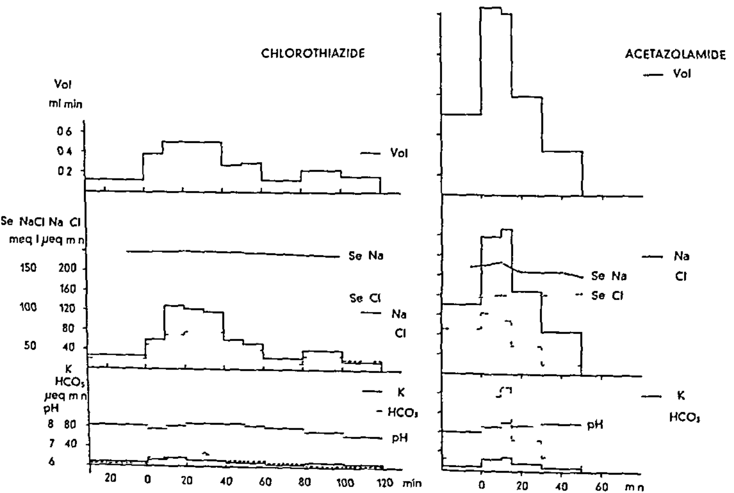
The urine volume rose to just over double during the first 10 minutes after the injection Then it fell steadily, to reach almost the initial value within 30 minutes

Sodium excretion rose two to three fold during the first diuresis period and then fell parallel with the diuresis Chloride excretion, on the other hand, increased only slightly, potassium excretion somewhat The considerable increase in bicarbonate excretion compensated for the "deficient" excretion of chloride The pH rose correspondingly

In this experiment the serum sodium level was seen to fall Further, a minor rise in serum chloride was noticed, indicating a tendency towards development of hyperchloraemia as a consequence of the loss of bicarbonate after injection of acetazolamide

Aminometradine

This drug proved to be somewhat toxic to rabbits Only one [illegible]
[illegible]
[illegible]
[illegible] period of anuria Fig [illegible] illustrates the experiment with 20 mg/kg intravenously



F g. 2 Rabb t no 277 Chloroth az de 20 mg/kg intravenously at t me 0
Rabb t no 234 Acetazolam de 20 mg/kg intravenously at t me 0

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Aminometradine

This drug proved to be somewhat toxic to rabbits Only one experiment with 10 mg/kg and one with 20 mg/kg intravenously could be completed In the others the animal died, either as a direct consequence of the injection or after a period of anuria Fig 1 illustrates the experiment with 20 mg/kg intravenously

The urine volume increased immediately and soon fell again The experiment with 10 mg/kg followed a similar course but the diuretic action was extremely slight

The sodium excretion followed the diuresis The same was true of the chloride excretion, which, however, in other experiments (on animals as well as human subjects) was more nearly equivalent to the sodium excretion than in the one illustrated here The potassium excretion was influenced for a short period only and to a small extent The bicarbonate excretion increased a little The urinary pH fell slightly throughout the experiment

Glomerular Filtration

The effects of the four diuretics on glomerular filtration is shown by table I With all of them a rise of creatinine clearance followed the administration of diuretics, but only after theophylline did this rise reach appreciable values The sudden increase in urinary flow after the injection of diuretics causes elution of a more concentrated urine, giving a clearance value for the first period that is 15–20% too high When the results are corrected for this experimental error, the glomerular filtration is seen to remain uninfluenced by acetazolamide, aminometradine or chlorothiazide Theophylline, on the other hand, caused a considerable rise in filtration rate, which doubled during the first period after the injection and then decreased steadily to normal values

Such increase in glomerular filtration rate after administration of theophylline has been demonstrated by several workers, among them GREEN *et al* (1949) Different theories have been advanced about this action, which is supposed to be due either to raised filtration pressure (RICHARDS 1924) or to increased renal blood flow (DAVIS & SHOCK 1949) or to altered glomerular permeability (CUSHNY & LAMBIE 1921) A vasodilatory action of theophylline, with a consequent increased renal blood flow, is the most likely explanation

Experiments on Rats.

Experimental

The experiments were conducted on male rats by the technique of KOBINGER & LUND (1959) and LUND & STORLING (1959)

Four groups of four rats were used in each experiment The fasting rats were placed in wire cages over polyvinyl funnels but were allowed water *ad lib* Three of the groups received the diuretic by mouth dissolved in physiological saline The fourth was a control group given the same quantity of physiological saline The urine was collected over a six hour period

Table I.

Creatinine clearance after intravenous *administration* of theophylline, chlorothiazide, acetazolamide or *aminometradine* to rabbits

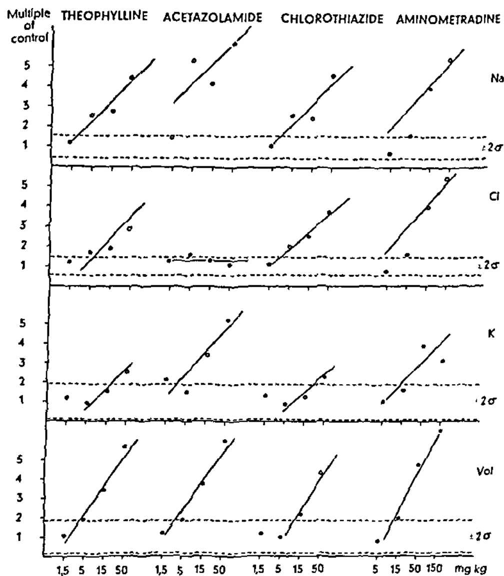
Clearance period no	*Dose mg/kg*	Theophylline		Chlorothiazide		Acetazolamide		Aminometradine	
		Diuresis *ml/min*	*Creatinine-clearance ml/min*	Diuresis ml/min	Creatinine-clearance ml/min	Diuresis ml/min	Creatinine-clearance ml/min	Diuresis ml/min	Creatinine-clearance ml/min
before inj	20	0.18	6.5	0.13	15.7	0.86	16.2	0.53	9.2
1	mg/kg	1.80	30.4	0.38	21.0	1.90	14.3	1.35	10.3
2	i v	5.50	22.0	0.50	11.9	1.80	11.8	1.25	11.9
3	inj	3.43	15.4	0.50	11.3	0.96	9.1	0.85	8.2
4		1.80	13.7	0.50	12.1	0.43	8.6	0.88	7.4
5		1.03	10.2	0.27	6.4	–	–	0.90	8.2
6		0.80	12.8	0.28	6.4	–	–	–	–
before inj	20	0.07	11.9	–	–	–	–	–	–
1	mg/kg	0.80	28.3	–	–	–	–	–	–
2	i v	2.60	14.9	–	–	–	–	–	–
3	inj	1.16	10.0	–	–	–	–	–	–
4		0.80	8.2	–	–	–	–	–	–
5		0.50	6.9	–	–	–	–	–	–
6		0.38	7.9	–	–	–	–	–	–
before inj	10	0.17	4.5	0.23	8.3	0.39	9.9	0.19	7.6
1	mg/kg	2.20	22.8	0.55	10.0	1.10	13.5	0.26	13.8
2	i v	1.11	7.0	0.71	9.5	0.80	10.4	0.15	8.9
3	inj	0.83	6.6	0.66	9.6	0.48	8.3	0.10	6.2
4		0.80	5.2	0.70	8.4	0.35	6.9	0.12	8.4
5		–	–	0.58	7.9	–	–	0.14	10.2

Results.

Doses of 1.5, 5, 15, 50 and 150 mg per kg body weight were given of each of *the four diuretics.*

To investigate whether the four diuretics were active after the first 6 hours, urine was collected during the next 6 hours in the experiments with 15 and 50 mg/kg. The values for urine volume and electrolyte excretion showed for all four drugs that their diuretic action must be regarded as exhausted within 6 hours of administration.

The results are set out and compared in fig. 3 and table 2, which roughly



was increased after orally administering four

Table 2

Factor by which normal excretion was increased after oral administration of 15 mg/kg of four diuretics to rats

Diuresis	Na	Cl	K	Vol
Theophylline	3 2	2.3	1	3 7
Chlorothiazide	3 1	2.7	1	2.4
Acetazolamide	5 2	1	3 7	3 9
Aminometradine	3 0	1 8	2.1	2.2
Control	1	1	1	1

reflect the mechanisms of action of the drugs Theophylline and chlorothiazide both acted diuretically by raising the excretions of sodium, chloride and water Acetazolamide showed a natriuretic action and also, unlike in the rabbit experiments, a pronounced kaliuretic action, whereas it had no influence on chloride excretion Our technique did not permit confirmation of an increased bicarbonate excretion instead Aminometradine raised equally the excretions of sodium, chloride, potassium and water, but the drug was not particularly active at the lower dose levels

Experiments on Human Subjects.

Experimental

[illegible]

taken

Theophylline

Three experiments were done Fig 4 illustrates an experiment with 500 mg theophylline

The urine volume increased during the first 12 hours of the experiment, but fell within the next 12 hours to only half the volume of the 24-hour pre period, a sign of the fluid retention after an "unnecessary" fluid loss The excretions of potassium sodium and chloride behaved similarly

The creatinine clearance was slightly raised during the first 12 hours

The bicarbonate excretion was raised slightly during the first three hour peri

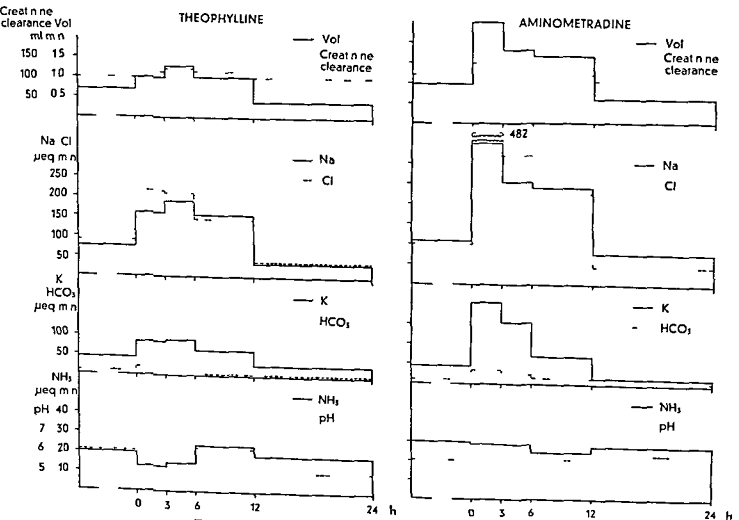


Fig 4 I S H 75 kg Theophylline 500 mg by mouth at time 0
P R. 68 kg Aminometradine 600 mg by mouth at time 0

The ammonia excretion plainly followed the variations in urinary pH It decreased with rising pH values, indicating active renal participation in the acid base regulation of the organism With rising pH and bicarbonate in the urine, the renal ammonia production was reduced as a compensatory measure

The serum electrolytes remained completely unaffected by the diuresis due to theophylline

Chlorothiazide

Experiments were conducted with ½, 1 and 2 g chlorothiazide Fig 5 illustrates the experiment with 1 g

The urine volume was increased during the first 12 hours and fell within the next 12 hours to somewhat below the volume of the 24 hour pre period

The creatinine clearance remained unaffected

The excretions of sodium and chloride also increased during the first 12 hours of the experiment The potassium excretion doubled in the course of the first 6 hours The drug also had a marked effect on the bicarbonate excretion for 6 hours In the experiment with 2 g chlorothiazide the potassium as well as the bicarbonate excretion remained raised for 12 hours

The ammonia excretion decreased with rising pH of the urine, but increased again towards the end of the experimental period, when the pH fell towards the original value

Serum bicarbonate dropped from 32 mEq/l to 26 mEq/l, but the remaining serum electrolytes did not alter significantly

Aceta-olamide

Two experiments were conducted with 0 5 g acetazolamide

In the experiment illustrated in fig 5 the drug is seen to have caused a considerable diuresis lasting throughout the 24 hours of the experiment The excretion of sodium and, especially, that of potassium increased correspondingly throughout the period whereas the chloride excretion remained practically unaffected The bicarbonate excretion, on the other hand increased considerably An appreciable compensatory decrease in ammonia excretion was observed

The creatinine clearance remained unchanged

Here too, serum bicarbonate only was seen to have altered significantly, this having dropped from 26 mEq/l to 22 mEq/l

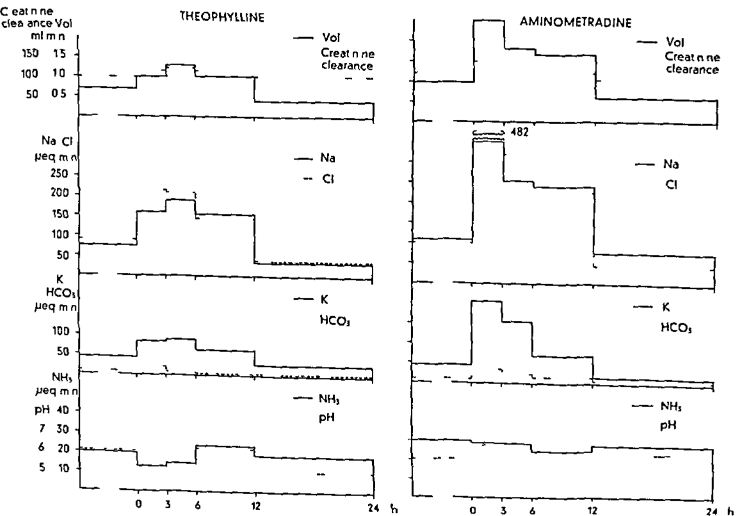


Fig 4 I S H 75 kg Theophylline 500 mg by mouth at t me 0
P R 68 kg Aminometradine 600 mg by mouth at t me 0

Aminometradine

Two experiments were conducted with 600 mg aminometradine Fig 4 illustrates one of these

The urine volume increased during the first 12 hours The excretions of sodium chloride and potassium likewise remained increased for 12 hours after administration of the drug A surprising observation was a considerably increased potassium excretion, also seen in the other experiment Consistently with this the chloride excretion increased relatively more than the sodium excretion

During the last 12 hours of experiment the urine volume as well as the electrolyte excretion were lower than during the 24-hour pre period, indicating an oliguric phase

The urinary pH and the ammonia excretion did not alter appreciably The serum electrolytes also remained unaffected

Discussion.

In *rabbits* the four diuretics all increased the flow of urine Theophylline had a prompt and powerful, but short lived, action, whereas chlorothiazide had a slower but more prolonged action Acetazolamide and aminometradine produced only a moderate diuresis in the rabbits Theophylline caused a pronounced and equal increase in the excretion of sodium and chloride Chlorothiazide produced a slightly less marked increase in chloride excretion but a greater increase in bicarbonate excretion Administration of acetazolamide led to a negligable increase in chloride excretion but a pronounced increase in bicarbonate excretion Potassium excretion remained largely unaffected by all four diuretics in these experiments However, no great importance can be attached to the results for potassium because even acetazolamide, which is known from the literature to have a strong kaliuretic action, had no essential influence on potassium excretion with our technique Acetazolamide was the only drug to have a marked action on bicarbonate excretion and urinary pH Theophylline was the only one of the four drugs that raised the glomerular filtration rate

It is difficult to estimate the relative potencies of the four diuretics on the basis of the *rat experiments* the drugs having different modes of action By attaching more importance to the sodium and chloride excretion and less to the volume (there having been free access of the animals to water during the experiments) we may argue that theophylline and chlorothiazide are about equally potent diuretics and aminometradine somewhat

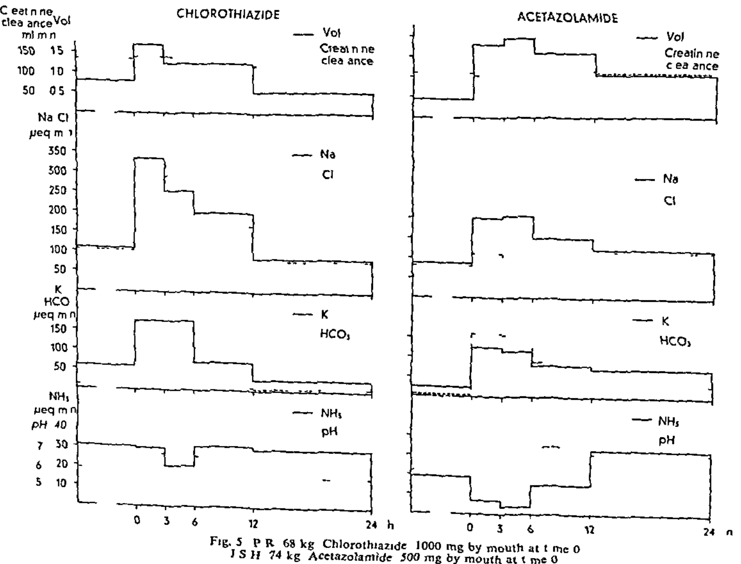


Fig. 5 P R 68 kg Chlorothiazide 1000 mg by mouth at time 0
J S H 74 kg Acetazolamide 500 mg by mouth at time 0

Aminometradine

Two experiments were conducted with 600 mg aminometradine Fig 4 illustrates one of these

The urine volume increased during the first 12 hours The excretions of sodium, chloride and potassium likewise remained increased for 12 hours after administration of the drug A surprising observation was a considerably increased potassium excretion, also seen in the other experiment Consistently with this, the chloride excretion increased relatively more than the sodium excretion

During the last 12 hours of experiment the urine volume as well as the electrolyte excretion were lower than during the 24-hour pre period, indicating an oliguric phase

The urinary pH and the ammonia excretion did not alter appreciably The serum electrolytes also remained unaffected

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weaker As stated above, acetazolamide is not directly comparable with the other drugs, but appeared in these experiments to be a powerful diuretic This comparison has been made on the basis of weights If, as would seem reasonable, we take account of the molecular weights of the drugs, which are of the order of 200, except chlorothiazide, whose molecular weight is about 300, we find that chlorothiazide had a somewhat more powerful action than theophylline in these experiments

The results of the *experiments on human subjects* confirmed in the main those from experiments on rabbits and rats Thus the excretion of electrolytes agreed approximately with the patterns of action demonstrated in the animal experiments, and the influence of chlorothiazide and especially of acetazolamide on acid-base balance was seen again in the experiments on human subjects In the latter there was also demonstrated an increased glomerular filtration as a response to theophylline One question has been further elucidated by the experiments on human subjects Theophylline and aminometradine were found to have little effect on bicarbonate excretion, whereas chlorothiazide, and particularly acetazolamide, greatly increased the excretion of bicarbonate, the latter drugs thus causing a fall in serum bicarbonate

The final evaluation of the four drugs as diuretics thus turned out in a sense to the advantage of theophylline, which was found to have the "purest" action on the excretions of water, sodium and chloride

Our investigations, especially those on human subjects, were, however, not sufficiently comprehensive to permit conclusions as to the clinical usefulness of the drugs

Summary.

A comparative study of acetazolamide, aminometradine, chlorothiazide and theophylline has been undertaken in experiments on rabbits, rats and healthy human subjects

In these experiments theophylline was found to be a powerful diuretic, acting promptly, which increased the excretion of water, sodium and chloride, without appreciably affecting potassium excretion or the acid-base balance Chlorothiazide and acetazolamide were likewise powerfully acting diuretics in these short-term experiments However, in contrast to theophylline, their mode of action was marked by increased excretions of bicarbonate and potassium, most pronounced with acetazolamide Aminometradine was the least active drug, but it did not affect acid base regulation Theophylline was the only one of the four drugs to raise the glomerular filtration rate

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Effects of Calcium-Binding Substances on Orally *Induced Chloralose* and Barbiturate Anaesthesia

By

Erling Sognen

(Received November 9 1960)

DYBING, DYBING & STORMORKEN (1952) have shown that the toxicity of red squill is decreased when it is given with any of the calcium-binding substances (Ca-b s) sodium silicofluoride, citric acid or oxalic acid This antagonism might be due to an action on the calcium balance of the organism, as the concentration of ionised calcium is supposed to be a factor of importance in the tolerance to drugs of the digitalis group, to which red squill belongs

Subsequent investigations (DYBING & SOGNEN 1960), however, showed that this effect of sodium fluoride, sodium oxalate or tetracemine (NFN) (= edetic acid BP = EDTA) can only be demonstrated when the Ca b s is given by mouth simultaneously with the red squill, but not if they are given simultaneously parenterally, or the one by mouth and the other parenterally The calcium-binding action of these substances is therefore believed to reduce the rate of intestinal absorption of red squill On the basis of this observation it seemed of interest to clarify whether or not a Ca-b s would also weaken the action of other orally administered drugs

Investigations in this Department showed that the plasma level of sulphanilamide, for instance, may be lowered by Ca-b s The same is true for alcohol (SOGNEN 1959), which, according to BERGGREN & GOLDBERG (1940), is absorbed from the intestinal tract solely by diffusion Preliminary unpublished experiments by WILSON & WIESEMAN's method (1954) indicate that sulphonamides also are passively transported (SMYTH, SNOWDEN & SOGNEN) By SHEFF & SMYTH's perfusion method (1955), the intestinal absorption of glucose, which is in part actively transported, has also been found to be depressed (SOGNEN 1959) Simultaneously with the above investigations, but totally independent of them,

GRASBECK *et al* (1958, 1959) found tetracemine to depress the absorption of vitamin B_{12} in man and calcium to raise the reduced absorption of vitamin B_{12} in cases of steatorrhoea and pernicious anaemia ABELS *et al* (1959) on the other hand, saw no reduction in vitamin B_{12} absorption by gastrectomised rats when the vitamin was given together with gastric juice from the rat and tetracemine doses of the same order as in our work

GRASBECK & NYBERG's, as well as ABELS', experiments seem to have been independent of each other and based on the observation that the increase in vitamin B_{12} absorption caused by hog intrinsic factor in preparations of liver and intestinal tissue depends on the presence of Ca^{++} and that this increase can be eliminated by adding tetracemine to the medium (HERBERT 1958, MINARD & WAGNER 1958)

In this work chloralose and mebumal (= pentobarbital) were given orally to observe possible changes caused by Ca b s in the course of the anaesthesia Diemal (= barbital) was chosen for studying the barbiturate level in plasma, because it is eliminated very slowly In these circumstances, and even if the animals are not nephrectomised, the plasma barbiturate level is more likely to bear relation to the intestinal absorption of diemal than to any other factors contributing to plasma level the first few hours after administration

Material and Methods.

Where not otherwise stated, full grown male albino rats, weighing from 200 to 280 g, and male rabbits ranging in weight from 2 to 4 4 kg were used

The rats had access to neither food nor water during the night before the experiment The rabbits fasted for 24 or 48 hours as recorded for the individual animals but had access to water

Chloralose mebumalnatrium (NFN) (the sodium salt of ethyl 1 methyl-butyl barbituric acid pentobarbital sodium (NNR), (in the text mebumal) and diemal natrium (the sodium salt of ethyl-ethyl barbituric acid barbitone (BP) = barbital (USP) in the text diemal) were given orally to the rats by means of a 5 ml record syringe to which a glass stomach tube was fastened

The rabbits were given the solutions specified through a rubber tube

The rats received 100 mg/kg chloralose in 1% (w/v) solution, 80 and 100 mg/kg mebumal in 0 8 and 1% (w/v) solutions and diemal 150 mg/kg in 1 5% (w/v) solution The rabbits were given 100 mg diemal per kg in 1% (w/v) solution Ca b s and the control substance sodium chloride magnesium chloride magnesium oxide or calcium chloride was given simultaneously with and in the same sol
in such amounts that the volu
rats or rabbits but in the rabt
duced afterwards to wash the

All solutions were made immediately before use and were administered at approximately body temperature

The effects of Ca b s on the chloralose and the mebumal anaesthesia were observed by comparing the course of the anaesthesia in the control and the experimental groups

The plasma barbiturate level was determined in blood samples withdrawn from rats by cardiac puncture and from the ear veins of the rabbits

For cardiac puncture a heparinised 2 ml all glass syringe and cannula no 12 were used

The blood samples from rabbits were taken by means of a heparinised Carlsberg constriction pipette, as described by SCHOU (1958) In a few experiments the coagulability increased considerably after some samplings in that events the samples were then drawn by means of a syringe

The blood was transferred by means of a constriction pipette to small test tubes containing 2 ml of 0 1% sodium citrate solution, in which the pipette was washed repeatedly The tubes were sealed and left for at most 12 hours before analysis by Lous' method (1950), based on the characteristic ultraviolet absorption spectra of the barbiturates After extraction with chloroform and transfer to a buffer solution of pH 10, the absorption was measured at this pH and at pH 2 at 240 mμ The difference gives a basis for calculating barbiturate concentration

Standard curves were plotted by adding known amounts of the barbiturate to a pooled sample of serum from eight rats and to a pooled sample from six rabbits

The only deviation from the procedure described by Lous was the use in our analyses of 1 ml of blood plus 2 ml of 0 1% of sodium citrate, instead of the 3 ml of whole blood

A Beckman D U spectrophotometer was used for the optical measurements

Results.

Fig 1 shows that 100 mg chloralose per kg produced deep anaesthesia for 2 to 5 hours The same dose of chloralose administered together with sodium fluoride exerted only a mild action on the animals, which were all, but one able to move throughout the observation period The same tendency was found in the experiments with sodium oxalate

In repeated experiments with 100 mg and with 80 mg mebumal per kg, sodium fluoride and sodium oxalate reduced the anaesthetic action (fig 2, 3)

It is noticeable that with the largest dose of mebumal (100 mg/kg) the anaesthesia induction time was sometimes practically equal in the experimental and the control group

By intraperitoneal administration of mebumal no protective effect was seen from sodium fluoride given in the same solution as the anaesthetic

Table I shows that the depression of absorption depends on the concentration of Ca-b s when each substance is considered individually

Equimolecular amounts were given of sodium fluoride, tetracemine and sodium oxalate Comparing in this way the potencies of the substances as absorption depressors, it appears that sodium fluoride and tetracemin had

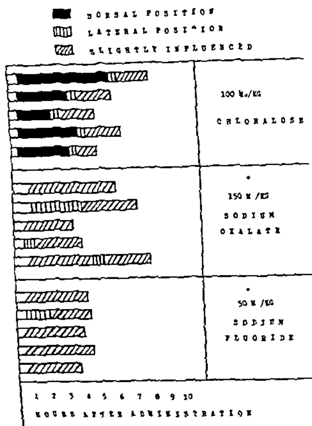


Fig 1 The effect of sodium fluoride and sodium oxalate on rate and duration of orally induced chloralose anaesthesia in male albino rats

approximately equal depressing effects on the absorption Sodium oxalate was a somewhat weaker depressor at the same concentration On the other hand comparing the substances on the basis of their calcium binding powers shows sodium fluoride, which is univalent, to be a more potent absorption depressor than tetracemin, which binds one molecule of Ca per molecule of tetracemin (provided the conditions are optimal) Oxalate is from this aspect also a somewhat weaker absorption depressor than either sodium fluoride or tetracemin It must be, however, emphasised that a better defined milieu would be required to justify considering the absorption depressing potencies of the various Ca b s in direct relation to their calcium binding powers After their intraperitoneal use no depression of absorption is seen

In rabbits absorption of barbiturates from the intestinal tract was likewise seen to be depressed by tetracemine (fig 4 and 5) After 24 hours

All solutions were made immediately before use and were administered at approximately body temperature

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Table 1

Ca b s mg/kg	n	Range µg/ml	Mean conc µg/ml	Per cent of controls
Sodium fluoride				
50	9	61–102	80	46 1
25	5	63–123	95	54.5
12 5	5	134–197	169	97 2
Tetracemin				
400	4	38–74	56	32 2
200	6	60–164	107	61 5
100	6	45–175	115	66 1
50	4	159–187	177	102
Sodium oxalate				
159	4	72–142	106	60 9
80	5	151–178	161	92 5
Controls	18	152 207	174	100
Intraperitoneal controls 100 mg/kg diemal	4	106–152	122	
+ 50 mg/kg sodium fluoride	4	107–125	125	–

of fasting and 400 mg tetracemine per kg, the depression was slight, whereas a marked depression was seen after 48 hours of fasting MAGEE & SEN (1932), after numerous experiments to study the influence of calcium on the absorption of glucose, state that the results varied considerably, and it was difficult to get any distinct picture Rabbits have a higher blood calcium level than rats

Absorption of Diemal from Solutions which are isotonic with or hypertonic in relation to Ca b.s Solution

As the control group and the experimental group in the above experiments received solutions of different osmotic pressures, it seemed of interest to examine whether the depression of absorption bears any relation to this difference

Therefore, in the next experiments 150 mg diemal per kg were given together with equimolecular amounts of sodium fluoride or sodium chloride Further, to discover whether a raised osmotic pressure depresses

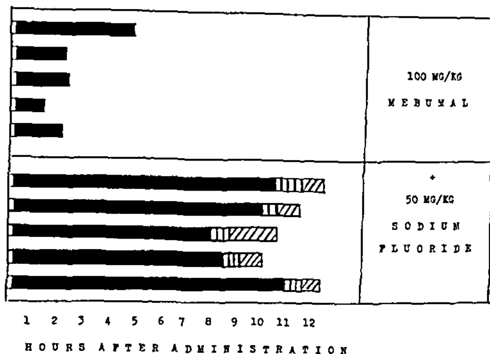


Fig 2 Effect of sodium fluoride on a lethal oral dose of mebumal to female rats

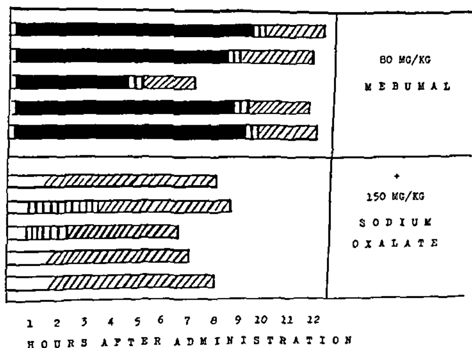


Fig 3 Effect of sodium oxalate on orally induced mebumal anaesthesia in male rats

Table 1.

The Effect of Sodium fluoride Tetracemin (edetic acid) and Sodium oxalate on Blood Concentration of Barbiturate (μg/ml) from Rats 1½ Hour after Oral Administration of 150 mg/kg Diemal (barbital)

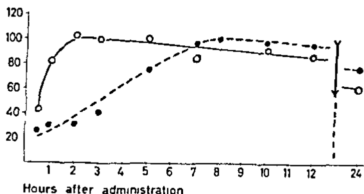
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Tetracemin				
400	4	38–74	56	32 2
200	6	60–164	107	61.5
100	6	45–175	115	66 1
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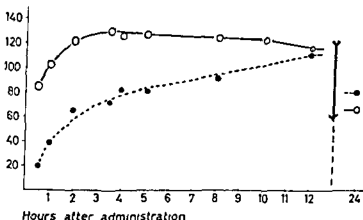


Fig 4 and 5 Experimental ●——● Control ○——○
Effects of 400 mg/kg tetracemine on the bloodconcentration of barbiturate (µg/ml) *after 100 mg/kg diemal orally to rabbits, fasted for 48 hours*

absorption at all, the same barbiturate dose was given together with relative large amounts of magnesium chloride and magnesium oxide We further examined whether calcium chloride in large amounts influences

Table 2

Concentration of Barbiturate (µg/ml) in Blood from Rats $1^1/_2$ Hour after Oral Administration of 150 mg/kg Diemal in Isotonic or Hypertonic Solutions in Relation to the Concentration of Sodium fluoride

Substance	Mg/kg	MEqv/l	n	Range µg/ml	Mean µg/ml
Sodium fluoride	50	238	9	61–102	80
Sodium chloride	69 5	238	4	188–196	193
Magnesium chloride	250	717	4	153–177	165
Magnesium oxide*)	250	–	4	164–185	175
Calcium chloride	250	675	4	154–161	164
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For the same reasons we may exclude the diuretic effect of NaF (WADDEL 1884) as the main cause of the low plasma level, because this renal effect should be the same, or even larger, after its parenteral administration

The course of the curve for plasma levels of barbiturate (fig 4, 5)

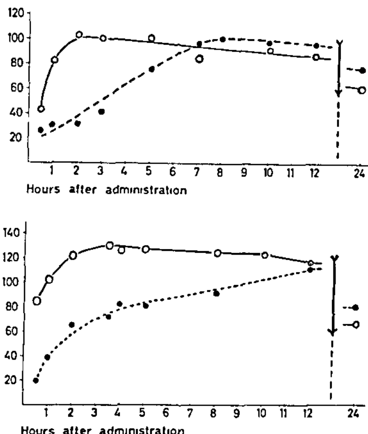


Fig 4 and 5 Experimental ●——● Control ○——○
Effects of 400 mg/kg tetracemine on the bloodconcentration of barbiturate (µg/ml) after 100 mg/kg diemal orally to rabbits, fasted for 48 hours

absorption at all, the same barbiturate dose was given together with relative large amounts of magnesium chloride and magnesium oxide We further examined whether calcium chloride in large amounts influences

Table 2

Concentration of Barbiturate (µg/ml) in Blood from Rats $1^1/_2$ Hour after Oral Administration of 150 mg/kg Diemal in Isotonic or Hypertonic Solutions in Relation to the Concentration of Sodium fluoride

Substance	Mg/kg	MEqv/l	n	Range µg/ml	Mean µg/ml
Sodium fluoride	50	238	9	61–102	80
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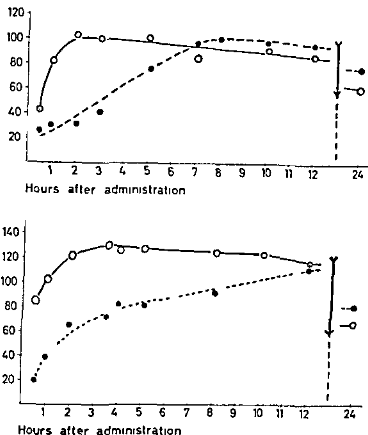


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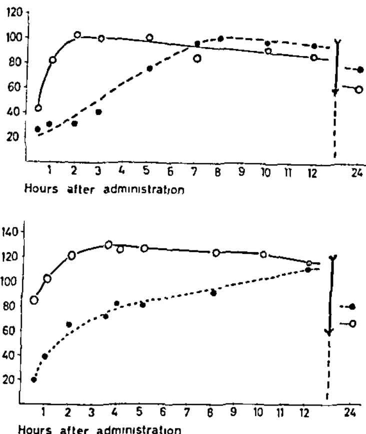


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likewise argues against such a mechanism, because in the experimental group the barbiturate concentration rose steadily for the first few hours after the administration. Finally, within the interval of 12–24 hours the barbiturate concentration was as a rule higher in the experimental than in the control group. Similar results were seen, and even more clearly, in experiments on rats given sulphonamide, when the falling curve for the control group intersected the rising curve for experimental group no later than after 6 hours (SÖGNEN 1959).

HENDRIX (1957) found a slower gastro-intestinal absorption of radioactive iodine from highly concentrated glucose solutions than from more dilute solutions. He took the rapid transport of fluid to the intestine when this contains a hypertonic solution to be the cause of the reduced absorption of iodine. The plasma level of barbiturate cannot be lowered by oral administration of diemal in solutions that are isotonic or hypertonic in relation to the Ca-b.s. solution (table 2). We can therefore leave out of account any direct osmotic effect of Ca-b.s. as the cause of depressed absorption.

In table 1 a comparison has been drawn between sodium fluoride, tetracemine, and sodium oxalate as depressors of absorption in relation to their calcium-binding potency. Sodium fluoride is seen to depress the absorption so effectively, compared with tetracemine and sodium oxalate, that its depressing action on the absorption can hardly be due to calcium-binding alone. It is probably related to the fact that sodium fluoride has an enzyme-inhibiting action, as demonstrated by various workers within widely different fields (SIEVERT & PHILLIPS 1959; LOHMANN & JENDRASSIK 1926 and many others).

HEIDENHAIN (1894) was the first to demonstrate that sodium fluoride depresses the absorption of sodium chloride from the intestinal lumen. He regarded this as due to competition between fluoride and the chloride ion for the routes of transport. Many workers have since used sodium fluoride to depress the active absorption of glucose from the intestinal tract. (No further account will here be given of this). The calcium-binding potency of the sodium fluoride has apparently not been regarded as responsible for the depression.

Several workers, whose results also will not be further discussed here, have studied the problem of the significance of calcium ion for glucose absorption (MAGEE & SEN 1931, 1932; GELLHORN & SKUPA 1933; MCDOUGALL 1935; GARDNER & BURGET 1938). The experimental condi- [illegible] were apparently contradictory. [illegible] in connection with the current investigation [illegible] on the factors determining the absorption rate from the intestinal tract.

Even if anaesthetics are suitable drugs for the purpo[illegible]

differences in pharmacological effect between experimental and control animals their use is not really convenient for the study of intestinal absorption as such

The secondary effects of anaesthesia may mask or accentuate differences in the absorption rate of the two groups It is therefore obviously a disadvantage that the control group is in state of deep anaesthesia, whereas the experimental group is only slightly affected

Accordingly non anaesthetising drugs are being used in the further investigation of this problem It has been shown that the absorption of several unphysiological substances is depressed by Ca b s , as also is the absorption of glucose and water (SOGNEN 1959)

According to SMYTH & TAYLOR (1955) and FISHER (1955) there is an inter relationship between absorption and metabolism of glucose and the water transport in vitro

Substances like glucose and fatty acids may be passively transferred in an active water stream (SMYTH & TAYLOR 1955, BARRY & SMYTH 1960) The same may to a certain extent be true of drugs

Our results therefore indicate that the inhibition of drug absorption caused by Ca b s may be due to depressed water transport, which again may be related to an effect on glucose absorption and metabolism

This and other possible explanations will be more closely discussed in papers being prepared for publication

Summary.

Oral doses of chloralose and mebumal (pentobarbital) that normally cause deep anaesthesia, or even death, are greatly weakened in their action when administered along with a calcium binding substance, such as sodium fluoride sodium oxalate and tetracemine (edetic acid) The plasma level of barbiturate has then been found much lower than in control animals Various criteria suggest that absorption from the intestinal tract is depressed

It has been shown that calcium binding substances also inhibit the absorption of glucose and the transport of fluid from the intestinal lumen

The results therefore indicate that the intestinal absorption of un physiological substances is related to the absorption of glucose and the transport of fluid

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From the Biological Department, The Pharmaceutical Institute,
University of Oslo

Zone Electrophoresis of Oxytocin by a Horizontal Column Technique

By

Kjell Briseid Jensen and Reidar Bredo Sund

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The moist chamber paper electrophoretic technique is not suitable for determining mobilities in the alkaline pH-region, as carbon dioxide will be absorbed into the buffer contained in the strips and the pH will accordingly decrease (SUND & BRISEID JENSEN 1960) The work presented here reports experiments in which the electrophoresis was accomplished in small-dimensioned polythene tubings to prevent a fall in pH and evaporation of solvent With a view to electrophoretic examination of human urinary kinin (GOMES 1955, WALASZEK 1957; BRISEID JENSEN 1958; GADDUM & HORTON 1959, HORTON 1959, BRISEID JENSEN & SUND 1959), presumably a polypeptide, the procedure was tested with a polypeptide of known electrophoretic properties, oxytocin, and also to some extent with vasopressin

1. Technique.

A Apparatus and Materials

Electrophoretic equipment LKB paper electrophoresis apparatus, type 3276 B,

Equipment for pH-measurements Metrohm pH meter type E 196 S (Metrohm AG, Herisau Switzerland)

Supporting materials Granular starch (Amylum Solani Ph N V) Powdered cellulose (Ashless Powder Chemically Prepared, Standard Grade, Whatman, W & R. Balston, Ltd , England)

Migrants In most experiments synthetic oxytocin was used (Syntocinon ® pro injectione, 5 I U per amp , Sandoz A G , Basel, Switzerland) The polypeptide was recovered from the ampoules by freeze drying

In some experiments a partly purified vasopressin was used, (Pitressin ® ampoules 10 I U per amp , Parke, Davis & Co , Hounslow, England), also recovered from the ampoules by freeze-drying

Indicators for electro-osmosis Digitoxose (L Light & Co , Ltd , Colnbrook, England) was generally used In some experiments methanol (A R , E Merck, A G , Darmstadt, Germany) or acetanilide (Ph N V) was employed

Chemicals were of A R quality throughout

B *Procedure.*

Pretreatment of supporting materials 10 g of *granular starch* (a quantity suitable for a single column, vide infra) were stirred in a beaker for 15 minutes with 40 ml of buffer and allowed to sediment for 15 minutes This was then decanted and the process was repeated twice The pH of the last washing was found to be slightly lowered when alkaline buffers of low capacity were used In those circumstances the buffer adhering to the starch was washed away with 40 ml of fresh buffer solution before packing the column

250 g of *powdered cellulose* were washed in a column with 10 1 0 2 N hydrochloric acid It was then washed with distilled water until the pH of the eluate was over 5 The powder was dried at 50° and sifted through a silk sieve with 30 threads per cm and apertures 0 23 mm (sieve VI, Ph N V)

Packing the column Polythene tubing, 60 cm long, was put into the stem of a 100 ml separating funnel (Quickfit D 2/22) and fixed by pushing it about 1 cm upwards The connection was secured by a 5–6 cm piece of tubing and a Mohr clip The polythene tubing was washed 2 or 3 times with buffer solution, and the buffer under suction was thereby forced up beyond the stopcock, which was then closed The lower opening of the tubing was closed with a rubber stopper, and the tubing anchored to a Microid shaker by means of 5–6 cm of tubing The latter was put into the hole in the top of one of the shaker arms and, after vertical stretching of the column, was slid over the column and fixed with a Mohr clip

The supporting material, which was used in excess, was transferred to the separating funnel by means of fresh buffer solution 10 g of starch or 4 5 g of cellulose powder were employed In experiments with acid or neutral buffers the solution was filled to a level about 2 5 cm above the opening of the separating funnel In experiments with alkaline buffers the 2 5 cm upper layer consisted of liquid paraffin (Ph N V)

The whole content of the separating funnel was set rotating by means of a glass rotor, and sedimentation was begun by partly opening the stop-

cock At the same time the Microid shaker was started, the shaking speed being adjusted to give the tubing a strong vibration Stirring and shaking were stopped by a "start and stop" clock – usually after 15 hours

Fitting the column into the electrophoresis apparatus The ready-packed polythene tubing was carefully removed from the sedimentation device and a rubber stopper put into the upper opening Then the lower 15 cm of the tubing were cut off and discarded At the point were the cut was made the contents of the tubing were dug out to a depth of about 2 cm and replaced with buffer solution A small piece of cotton wool (about 8 mg), which had in advance been soaked in the buffer and was free of airbubbles was then put into the tubing to prevent any movement of the supporting medium used

The polythene column was connected to the electrode vessels by Pyrex glass tubing (int diam 2 8 mm, ext diam 4 3 mm) bent at right angles, with arms 3 and 7 cm long The short arm was filled with buffer solution and put into the cut end of the polythene tubing to a depth of about 0 8 cm With the tubing resting in a horizontal position a new cut was made 26 cm from the first one, and the other glass tube was fitted in as described above Then both the angled glass tubes were completely filled with buffer solution, care being taken to avoid air bubbles The column was stretched out horizontally on the cassette floor, resting on 4–5 small pieces of rubber tubing, and then secured with cellulose tape The cassette floor could now be placed in its position between the electrode vessels, which had in advance been filled with buffer solution Usually 650 ml were used in each vessel In experiments with alkaline buffers the solutions in the electrode compartments were covered with liquid paraffin, 125 ml per compartment In all runs to be compared the final volume was the same throughout Th [illegible]

[illegible] osmotic indicator in the buffer, both substances being thus applied at the same point Up to 50 µl were applied and this was also the most frequent volume The tubing was perforated with a cannula 3 to 6 cm from the anodic end of the supporting medium, and a canal was opened right through the tubing Then the test solution was applied by means of a syringe and a new cannula (a cannula not blocked by granula of the supporting material) Care was taken to prevent air from penetrating into the tubing and after application the hole was closed with silicon grease

Electrophoresis As soon as the test solution had been applied, electrophoresis was begun Total output was 80–200 V, usually 100–120 V Field strength was found by voltage measurements over a 20 cm central section of the column both at the beginning and at the end of the experiment As secondary electrodes 2 platinum rods (3 cm long and 0 6 mm

thick) with the lower end tapered were used These electrodes were removed during the run and the perforations closed with silicon grease Field strengths for the buffers in table 1; cellulose (100 V). about 2 0 V/cm and 3/4 mA/tubing; starch (120 V)· about 3 6 V/cm and 1/2 mA/tubing An 18–25-hour electrophoresis time was used for the oxytocin experiments The room temperature was kept fairly constant, in the oxytocin experiments 23° ± 2°

Table 1.

Electrophoretic and electro osmotic mobilities (cm/sec/volt/cm) on powdered cellulose or starch

Migrant Oxytocin, concentration in test solution 10 units/ml
Electro osmotic indicator Digitoxose, concentration in test solution 3 5–8 0 mg/ml
Voltage 100–120 volts
Duration of run 18–25 hours
Temperature 23° ± 2

Concentrations of barbital sodium and sodium chloride were kept constant (0 003 M and 0 047 M), pH was varied by adding different quantities of free barbital
The figures are average values of 2–4 runs

Barbital M	pH	Van Slyke buffer values	Powdered cellulose		Starch	
			$\mu \cdot 10^5$	μel osm $\cdot 10^5$	$\mu \cdot 10^5$	μel osm $\cdot 10^5$
0 0300	7 00	0 0062	−0 02	4 1	1 1	1 5
0 0105	7 40	0 0054	−0 5	4 4	0 7	1 6
0 0045	7 75	0 0041	−0 8	4 6	0 5	1 7
0 0020	8 15	0 0028	−0 8	5 1	0 3	1 7

Estimation of distance travelled After the run, the column was cut into 1 cm pieces, which were shaken out, filtered and analysed for content of migrant and electroosmotic indicator In the first experiments we cut the tube with a razor-blade, but later we used a cutting apparatus devised by O Gjessing, The Chemical Institute, University of Oslo (fig 1) The column is put into the narrow channel on the left side of the apparatus and gently pushed beneath the knife to the bottom of the shallow groove in the end of the horizontally placed adjustment screw The screw can be kept firmly in position by means of a "braking" screw, and it can be adjusted to give pieces of tube from a few mm up to 20 mm in length

In the experiments with oxytocin or vasopressin, digitoxose was used as electro-osmotic indicator The cut pieces of column were then shaken directly with 2 5 ml de Jalon's solution and filtered through cotton wool in shortstemmed glass funnels Aliquots of 0 5 ml were pipetted off for determination of the sugar and the residual solutions were used for bi-

ological assays The migration distance was defined as the difference in linear displacement between the peak of the migrant and the peak of the indicator Mobilities were expressed as cm/sec/volt/cm

Quantitative determinations The *oxytocic activities* of both oxytocin and vasopressin were assayed on the isolated rat uterus, essentially as described previously (BRISEID JENSEN 1958) Matching was done by bracketing" the unknown fractions with two doses of standard differing by a factor of 1 33 An isotonic frontal writing lever was used (SCHILD 1947), and the bath volume was 10 ml As standard preparation the same substance was used as that submitted to electrophoresis

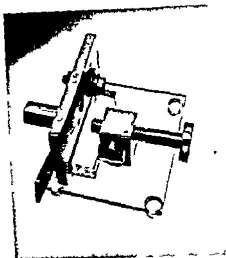


Fig 1 Apparatus for cutting columns For description see text

For determination of *digitoxose* 2 00 ml ca 12 N hydrochloric acid was added to
as measured after 1 hour or more, at room
with
ectro-

llows
phoric
mixture was
kept at room temperature for 10 m
s of perman
ganate was eliminated by adding a saturated solution of sodium pyrosulphite from a burette that could deliver small drops The decolorising – being critical for the deter-
Concentrated sulphuric acid 4 00 ml was

bath After cooling down to room
is
added to10 00 ml and the colour measured at the absorption maximum – filter M
Photometer being used

e at 238 mμ on a Beckman Spectro-

f the readypacked columns the flat
ws The contents of a 2–3 cm piece of
with a glass rod to fit the membrane
al and fixed in position when the

close to the flat membrane electrode and kept in pos
The calibrations were done with the electrodes immersed in the standard solutions

2. Comments on the Technique

Packing the column A sedimentation time of 15 hours was always sufficient for complete filling of the polythene tubing, and the supporting material was never found to be further compressed after that The adjustment of the stopcock of the separating funnel was critical for successful packing and required some practice The speed of the rotor and the Microid shaker usually had to be adjusted after some running time To estimate the reproducibility of the packing procedure several pieces of tubing were packed with powdered cellulose and 0 1 N sodium hydroxide Each one was cut into 5 cm pieces, which were shaken with 10 ml portions of water and titrated with 0 01 N hydrochloric acid The average titration value of 8 52, with a standard deviation 0 18, indicates both even distribution of supporting medium and electrolyte within the individual pieces of tubing, and satisfactory reproducibility of packing However, the deviations observed in electro-osmotic mobilities were far more pronounced (vide infra Estimation of distance travelled)

The pieces of tubing were visually inspected before use A few pieces had to be discarded owing to evidently unsuccessful packing with gaps in the supporting medium

Test solution To obtain the same field strength in the section containing the migrant as throughout the column, the additional conductivity of the test solution due to the presence of the migrant must be negligible This will happen when, for example, a neutral salt such as sodium chloride is the main ionized substance of the buffer and the concentration of migrant is so small compared to concentration of buffer substance that the pH of the test solution is the same as the buffer pH without addition of strong acid or base The latter requirement must also be fulfilled in order to know the true pH during the whole electrophoretic run In preparing test solutions the above mentioned suggestions were taken into consideration The oxytocin solutions were accordingly made up at a concentration of 5–10 units per ml (0 01–0 02 mM) Electrophoretic mobilities being to some degree dependent on the concentration of the migrant, the same concentrations were used in experiments to be compared and also the volumes applied were as nearly equal as possible

As vasopressin posseses only a weak oxytocic effect, and as the vaso pressin preparation employed was not highly purified we had to use a fairly strong concentration of the substance The pH accordingly fell and had to be re adjusted with sodium hydroxide Mobilities for vasopressin are therefore not given

The digitoxose was used at various concentrations, depending on the photometer employed (3 5 mg/ml for Beckman B–8 0 mg/ml for Coleman

Junior 6 A) The differences in digitoxose concentration did not seem to affect the electro osmosis

Experiments with coloured solutions demonstrated that the test solution would completely fill the square area of the tubing and diffuse to both sides of the application point When cellulose powder was used as supporting material, it absorbed the test solution in such a way that the applied fluid could not be observed The starch, however, was less elastic and a small section with fluid normally appeared beneath the application-hole

Electrophoresis The voltage range used was based on experiments in which the temperatures of the central parts of the columns were determined For these measurements a thin-threaded constantan-copper thermo element was employed, connected to a highly sensitive mV-meter With some buffers of ionic strength 0 05 and an overall voltage of 200 V, the rise in temperature did not exceed 2° With increasing voltages the rises became considerable, up to 10° was observed for 350 V

The smallness of the current passing the columns necessitated the use of a vacuum tube voltmeter for voltage measurements The polarization of the platinum rods used as secondary electrodes must be insignificant (SCHILLING & WALDMANN-MEYER 1959) and, anyhow similar in runs carried through under essentially the same conditions

In the cellulose columns a small increase in voltage from the beginning of electrophoresis to the end was usual, in the starch columns, however, a small decrease was observed Voltage measurements of successive 5 cm sections of a starch column showed that the decrease was the same throughout, within the error of measurement

The average value of the measurements at beginning and end of a run was chosen for calculating potential gradients during the run For both types of supporting material the difference between the two values usually fell between 2 and 6% In a single run we noticed a voltage fall of 33% in a starch column, which was accordingly discarded In two columns prepared with "soluble" starch (Amylum solubile p a Merck) the decreases in voltage were 28% and 52% Microscopic examination revealed an enlargement of the starch granules during the run, which makes it probable that the voltage change may have been due to solution of the material having begun

Estimation of distance travelled Mono- as well as polysaccharides have been widely used as indicators of electroosmosis From a theoretical point of view a substance like hydrogen peroxide might have been preferable (Ciba Foundation Symposium on Paper Electrophoresis, London, 1956, General Discussion, 213) Its reactivity, however, severely limits its use Amino acids, for example, will be oxidised to the corre-

sponding keto acids We chose to use digitoxose as indicator, a desoxy-sugar easy to determine quantitatively In some preliminary experiments with cellulose powder and acetate buffer of pH 5 5 the movement of digitoxose was compared with those of acetanilide and of methanol Digitoxose and acetanilide could be applied together, and a few runs were sufficient to reveal that the sugar moved faster than the other substance, which was probably delayed by adsorption Digitoxose and methanol had to be run in different columns, as the sugar to some extent influenced the determination of the alcohol With 10 columns the average mobility for digitoxose was 2 78 10^{-5} (s = 11 1%) and with 11 columns the corresponding value for methanol was 2 87 10^{-5} (s = 8 4%) The difference was not significant

Reference has previously been made to titration experiments suggesting that the sedimentation method used gave uniform columns However, the variations that could be observed for electro osmotic mobilities far exceeded those of the titration results Even if several other factors contribute to this effect (variations in temperature, error in judging the peak of indicator, error in the estimation of potential gradient and so on), the considerable deviations found in electro osmosis could also be due to differences in the packing of columns, not detectable by the titration method, which might strongly affect mobilities

The number of parallel runs at each pH was made dependent on the observed deviations in electro osmotic mobilities Accordingly, the larger error found for the average electrophoretic mobilities must to a large degree depend upon the error of the biological assays

In both types of supporting material the oxytocin could be recovered in quantities sufficient for assay over 3 to 4 sections (except when tailing occurred), the peak being found in the central section The method employed for determination of digitoxose rendered possible an estimation of weaker side fractions also

The distance travelled by a substance was estimated by the peak position, and the peak was calculated as the point of 50% distribution in a cumulative curve This method of calculation presupposes a normal distribution for the quantitatively most important fractions However, a deviation from normal distribution that is practically the same from one pH-value to another still makes a comparison possible *within* a buffer system, for though the mobility curve will be to some extent displaced, the calculated pI will also be displaced

The distributions of oxytocin and digitoxose on starch were obviously not normal for all pH-values examined Thus oxytocin displayed a tailing that increased with pH and was limited at pH 7 0, but considerable at pH 8 9 Digitoxose, on the other hand, gave a slight forward "tailing"

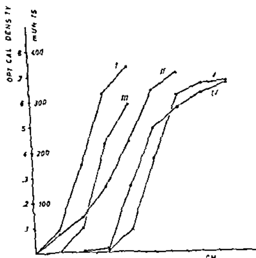


Fig 2 Distribution of oxytocin and digitoxose after electrophoretic run
Examples of cumulative curves

Abscissa Cm run. Only the shapes of the curves and not their actual positions after the run are given

Ordinate For oxytocin mU in 1 cm fractions For digitoxose the optical densities observed when one fifth of the eluate from every 1 cm fraction (0 5 ml) was added to 2 0 ml of hydrochloric acid reagent.

I Oxytocin on starch, pH 7 0
II Oxytocin on starch, pH 8 9
III Oxytocin on powdered cellulose pH 7 8
IV Digitoxose on starch, pH 7 0
V Digitoxose on powdered cellulose, pH 7 0

Fractions obviously due to tailing were omitted in the calculations from the cumulative curves Tailing complicated seriously any estimation of mobilities at higher pH-values (> 8 2), especially as the runs for the polypeptide examined at the same time became shorter

On cellulose columns no tailing was observed with the quantities applied In fig 2 are shown some typical cumulative distribution curves

Quantitative determinations The error of the biological assays must be the main factor contributing to the deviations in the measured electrophoretic mobilities To reduce the error, only sensitive (2 mU or less per ml bath) and stable uteri that discriminated satisfactorily between doses in the ratio 4 3 were used Several muscle preparations that could not fulfil these requirements had to be discarded If the activity could not be determined on the first d[illegible]

a day or two a[illegible]

For some b[illegible] substance at the concentrations used would influence the assays This was done by

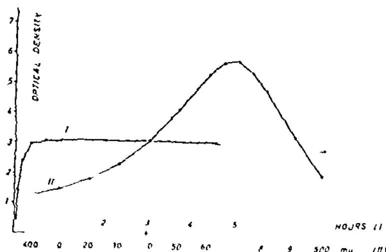


Fig 3 The digitoxose hydrochloric acid reaction
2 00 ml concentrated hydrochloric acid (about 12 N) mixed with 0 50 ml of solution of digitoxose in water

I Optical density at 470 mμ against time for 15 μg digitoxose read on a Beckman Model B Spectrophotometer 1 cm light path

II Optical density against wave length after complete development of colour read on a Coleman Junior 6 A Spectrophotometer with cuvettes 6 310 80 μg of digitoxose

assaying the active fractions against standard substance as usual, and at the same time against standard substance added to "blind" eluates from the column These eluates were obtained by shaking out sections without activity in the usual way In no instance could an effect of barbital on the height of concentration be positively established

As is evident from curve I in fig 3, the yellow colour in the determination of digitoxose increased in intensity for about 1 hour at room temperature The colour was stable The samples kept in darkness overnight lost only about 10% in optical density Fig 3 also shows the essential parts of the absorption curve (curve II)

Standard curves are given for both Coleman Junior 6 A (cuvettes 6–310) and Beckman B (1 cm light path) (fig 4)

pH-measurements The pH-measurements on the column contents showed good reproducibility and were easily made when powdered cellulose was the stabilizing medium Starch offered some difficulties owing to the "dryness" of the starch buffer mixtures In spite of some error occurring above pH 10, electrode EA 127 X was also used above this pH-value, and not only in packed material, but also for the sake of comparison for measurements with the electrodes immersed in buffer solution Even under conditions that should protect the buffer from the effect of atmospheric carbon dioxide, it was noticed that the readings on packed material were lower than those on the corresponding buffer solutions The fall in pH, however, seemed largely due to a "volum

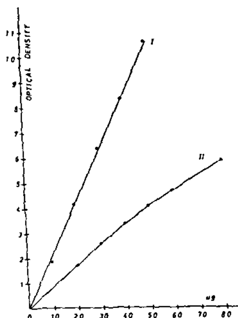


Fig 4 The digitoxose hydrochloric acid reaction
Standard curves based on readings from a Beckmann Model B Spectrophotometer, 1 cm light path (I) and from a Coleman Junior 6 A Spectrophotometer with cuvettes 6–310 (II) Procedure as stated in legend to fig 3

to an effect of the supporting material itself, as previously assumed (SUND & BRISEID JENSEN 1960) For example, the pH of a glycinate buffer (Γ/2 0 05) solution was found to be 11 60, whereas measurements on the packed material gave 11 45 The same pH-value was found when 50 μl drops were used

On the columns the alkaline buffers were protected against the effect of carbon dioxide A layer of an electrically indifferent fluid was used to protect the buffer solution in the separating funnel during packing and in the electrode compartments during electrophoresis Such a fluid should also have a low solubility in water and a low specific weight compared to the buffer solutions, and it should not dissolve the buffer substances A common solvent possessing the qualifications mentioned was liquid paraffin Many experiments showed that the technique described succeeded in keeping the pH unchanged As a buffer especially apt to be influenced by carbon dioxide we examined MILLER & GOLDER's (1950) pH 12 glycinate buffer, adding sodium chloride to ionic strength 0 05 The buffer capacity cannot be calculated on the basis of Van Slyke's formula, but was found experimentally to parallel 0 005 hydrochloric acid equivalents

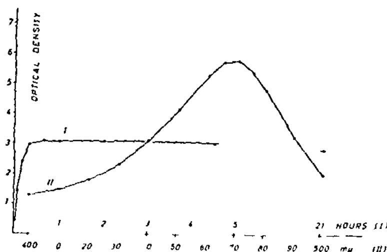


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barbital sodium and pH values down to pH 7 restricted the concentration of the salt to 0 003 M To make differences in conductivity of the test solution and the buffer negligible, a relatively large amount of sodium chloride, 0 047 M, was added By addition of different quantities of free barbital the pH was varied A pH value of about 8 15 represents the upper limit of the system's efficiency

It is evident from table 1 that the two different stabilizing media used gave markedly different results whereas the pI value of oxytocin on powdered cellulose was close to pH 7 0, the experiments on starch indicated a pI value > pH 8 15 KUNKEL, TAYLOR & DU VIGNEAUD (1953) found a value of 7 7, using paper strips as supporting medium (5°)

Some electrophoretic runs were made on powdered cellulose with a somewhat different barbital buffer system The ionic strength was 0 05, but the buffers were prepared by adding different quantities of hydrochloric acid to the same quantities of barbital sodium The results, given in table 2 confirm the low pI value of oxytocin on powdered cellulose

Table 2

Electrophoretic and electro osmotic mobilities (cm/sec/volt/cm) on powdered cellulose

Migrant Oxytocin concentration of test solution 6 7 units/ml and 5 0 units/ml in the experiment at pH 8 85
Electro osmotic indicator Digitoxose concentration in test solution 8 0 mg/ml
Voltage 100 volts
Duration of run 21–23 hours
Temperature 23 2
The figures are average values of 2 runs

Barbital sodium M	Hydrochloric acid M	pH	μ 10⁵	μ el osm 10⁵
0 05	0 034	7 65	−0 6	4 2
	0 026	8 00	−0 6	4 3
	0 013	8 45	−1 0	5 0
	0 005	8 85	−1 4	5 2

Starch was also used as the supporting medium for experiments with oxytocin in a barbital buffer system (ionic strength 0 05) that had a satisfactory capacity over the pH range 7 9–8 9 The polypeptide still seemed to move cathodically at pH 8 3, and with a rate nearly the same as at pH 8 15 in the buffer system of table 1 At higher pH values a

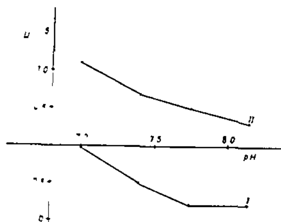


Fig 5 'Mobilities' for oxytocin on powdered cellulose (I) and on starch (II) Corrections for electro osmosis made by means of digitoxose
Experiments conducted in a system of barbital buffers, $\Gamma/2 = 0\ 05$, consisting of 0 003 M barbital sodium and 0 047 M sodium chloride pH varied by adding different amounts of free barbital
For further details see legend to table 1

per litre Measurements of pH were made 1) on the freshly prepared buffer (11 35), 2) on samples withdrawn from the separating funnels after packing (11 37), 3) on sections from the packed column (11 25) and 4) on samples withdrawn from the anode- (11 39) and the cathode compartments (11 40) after electrophoresis for 17 hours at 100 V Other experiments showed that the packed material kept its pH unchanged during electrophoresis

On running several columns together in the same apparatus the discharge at the electrodes can become so strong that the pH is altered throughout the compartments in spite of the baffles present In order that electrolytic products should not contaminate the columns, only one column was run in the apparatus when the buffer capacity was small

3. Results.

Table 1 and fig 5 give results from comparative electrophoretic experiments with oxytocin on cellulose powder and on starch It is evident from table 1 that the electro-osmosis on cellulose was considerably larger than on starch Barbital seems to be the buffer substance most used in the pH-area where the pI value of oxytocin is situated, and barbital buffers have also been used for paper electrophoretic estimation of this value (KUNKEL, TAYLOR & DU VIGNEAUD 1953) In our work barbital was used in spite of the rather low solubility of the free acid at the lower pH-range The combined requirement for buffers with a constant concentration of

barbital sodium and pH values down to pH 7 restricted the concentration of the salt to 0 003 M To make differences in conductivity of the test solution and the buffer negligible, a relatively large amount of sodium chloride, 0 047 M, was added By addition of different quantities of free barbital the pH was varied A pH value of about 8 15 represents the upper limit of the system's efficiency

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Vasopressin, too, seemed to give a lower pI value on powdered cellulose by the technique used than in corresponding previous investigations (10.9 for argininevasopressin, TAYLOR, DU VIGNEAUD & KUNKEL 1953). The vasopressin experiments were done in a glycinate buffer of ionic strength 0.05. The tailing of the polypeptide was considerable.

4. Discussion.

The influence of air carbon dioxide, which limits the use of the moist chamber electrophoretic technique for mobility experiments in the alkaline pH region, is abolished in the polythene tubing method described. At the same time evaporation is prevented, at least from a practical point of view, a matter of particular importance in constant voltage experiments.

However, our experiments have emphasized the additional problems arising when an attempt is made to use electrophoresis on supporting media for mobility experiments. The results clearly show that large differences in pI values are obtainable on different stabilizing media in spite of the remaining external conditions being kept constant. In our experiments corrections for electro osmosis were obtained by using digitoxose as indicator. It is obvious that several conditions relating both migrant and electroosmotic indicator to the supporting medium must be fulfilled to give pI values equal to those found on free electrophoresis. (Some authors give corrections for several factors, in order to convert the observed mobilities completely to free electrophoresis results). Even if this means that the term pI in zone electrophoresis can be dubious, pI determinations in such experiments still have use. Under well defined working conditions such values represent constants that will vary with the factors usually influencing pI values (buffer substances, buffer compositions, ionic strenght and so on).

Some workers have suggested eliminating the effects of the various factors influencing during zone electrophoresis by using a reference migrant, whose mobility is known from moving boundary experiments under otherwise identical conditions. However, in such experiments the difficulties mentioned above are also present, several requirements for the mode of interaction between migrant and reference migrant on the one hand and the supporting medium on the other must be fulfilled to obtain free electrophoresis pI results.

The usefulness of the method here proposed still needs further exploration. The precision of the method, judged by the standard deviation of the electro osmotic mobility of digitoxose, was not very satisfactory, being of the order of 10%. The precision of the electrophoretic mobilities must accordingly be still less. There may be general reasons for the magnitude

of error, for example, uneven packing of the column and variations in temperature, but factors specific to our experiments may also contribute Thus a better supporting material for the particular substance tested, oxytocin, might be found Starch gave considerable tailing at higher pH values, and cellulose gave low pI values, indicating reversible adsorption The distribution of digitoxose was to some extent also abnormal on starch, this fact adding to the difficulties of estimating the distance travelled at higher pH values A more suitable buffer system might also help in decreasing error The small buffer capacity of the barbital system used restricted the quantities of oxytocin, and this in turn led to rather flat distribution curves for it

As the method offers a considerable advantage for experiments in strongly alkaline buffers, and as it seems possible to reduce to various degrees the significance of the complicating factors just mentioned, further experiments should be carried out to improve the details of the technique and clarify its possibilities

Summary.

A zone electrophoretic method with horizontal polythene columns of small dimensions has been developed The technique described prevents the fall in pH in highly alkaline buffers that complicates the use of moist chamber paper electrophoresis

The method has been tested in a weakly alkaline pH region, with oxytocin as migrant, digitoxose as electroosmotic indicator and powdered cellulose or starch as supporting medium The polypeptide fractions were assayed on the isolated rat uterus, the digitoxose determined photometrically and the linear displacements of the substance directly used for calculation of "mobilities"

The pI value found on one supporting material were markedly different from that on the other (corresponding to pH 7 0 on cellulose powder and ~ pH 8 15 on starch) with the same barbital buffer system Neither powdered cellulose nor starch proved satisfactory as supporting material for oxytocin

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Experiences with a Fluorometric Method for Determining Corticosteroids in Man and Rat

By

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The determination of minute or rapid changes in adrenocortical activity by studies of plasma corticosteroid level would seem more meaningful than studies on urinary steroid output Determination of adrenal steroids in plasma may be performed by the colorimetric method of PORTER & SILBER (1950) This method of detecting 17, 21-diol 20-one steroids determines hydrocortisone (cortisol), which accounts for the greater part of corticosteroids in man (BUSCH & SANDBERG 1953, PETERSON 1957) and cortisone, but not corticosterone, the essential steroid of rat plasma (BUSCH 1953)

The specificity of the method has been criticised on account of the occurrence of the non specific Porter-Silber chromogen (MARKS & LEFTIN 1954, BUSCH & SILBER 1955) Large quantities of plasma are needed for the determination because of the method's relatively low sensitivity The fluorometric measurement of plasma corticosteroids may be regarded as preferable in several ways It was first proposed by SWEAT (1954) and later investigated by KALANT (1958) By its means principally corticosterone and hydrocortisone show pronounced fluorescence in strong sulphuric acid, the peak being situated at an activating wave length of about 4700 Å and a fluorescent wave-length of about 5500 Å

A column chromatographic technique has been described to distinguish between hydrocortisone and corticosterone (SWEAT 1954, SWEAT *et al* 1953) Without this separation, however, the results can still be of interest Such simplified modifications of the method have been [illegible] By these [illegible] –0 2 ml)

The aim of our investigation has been, first, to confirm and extend the

findings of previous studies on fluorometric determinations of plasma corticosteroids and, secondly, to adapt the method for routine clinical work and for determinations of rat plasma corticosteroid in pharmacological studies.

Method.

The method is essentially that given by GUILLEMIN *et al.* (1959), but certain *modifications* have been made:.

1. Determinations were done on 0 25 ml plasma samples from man or rat
2 For rat plasma samples, corticosterone standards of 0 05 and 0 1 µg have been used for comparison, for human plasma samples, standards of 0 1 and 0 2 µg of hydrocortisone
3 As fluorescence medium, a mixture of concentrated sulphuric acid and ethanol 75/25 (v/v) was used (ZENKER & BERNSTEIN 1957, DE MOOR *et al* 1960) Corticosterone and hydrocortisone give higher fluorescence in this medium than in the original 30 N sulphuric acid (vide infra)

Blood samples from the rat were taken mostly by decapitation, sometimes by bleeding from the jugular vein, into glass tubes, prepared with a few granules of heparin The animals were anaesthetized mostly with ether, sometimes with pentobarbital*) Blood samples from man were drawn by venipuncture, the tubes being prepared with heparin Heparin does not seem to interfere with the measurement of corticosteroids by this method (GUILLEMIN *et al* 1958)

Experimental

The fluorescence readings are directly proportional to the steroid concentrations for the range used in this method, as can be seen from the calibration curves (figs 1 and 2) Deviations from the straight line do not

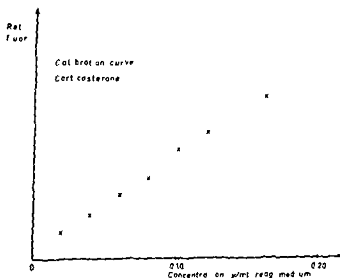


Fig 1 Concentration fluorescence curve, corticosterone

*) Mebumal (NFN)

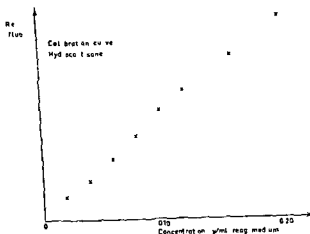


Fig 2 Concentration fluorescence curve hydrocortisone

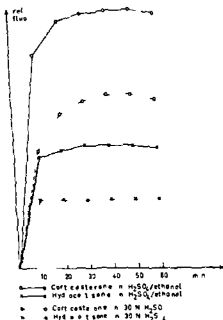


Fig 3 Development of fluorescence of corticosterone and hydrocortisone in 30 N sulphuric acid and sulphric acid/ethanol mixture

occur until concentrations above 6 5 µg of corticosterone per ml of 30 N sulphuric acid are reached (SWEAT 1954)

The relation between hydrocortisone and corticosterone in two different reagent mediums has been studied As can be seen from fig 3, these

steroids in sulphuric acid/ethanol medium give higher readings than in 30 N sulphuric acid, thus making it possible to use the spectrofluorometer at a more reliable range of sensitivity with lower blank readings The sensitivity of the method will also be somewhat improved In 30 N sulphuric acid hydrocortisone gave readings amounting to 32% of that of corticosterone, which is in accordance with the figure given by SILBER *et al* (1958) In sulphuric acid/ethanol reagent medium the corresponding figure was found to be 47%

In this experiment, it was observed that the development of the fluorescence was complete at a later time for corticosterone than for hydrocortisone, irrespective of the fluorescence medium

Results and Discussion.

The results of this investigation have been tested in accordance with the criteria of reliability given by BORTH (1956) and DICZFALUSY (1957)

Precision

The precision of the method can be assessed by calculating the estimate of standard deviation (s) from the difference between the two results of duplicate determinations, *according to the formula* $s = \sqrt{\frac{S(d^2)}{2N}}$ *where* d is the difference between the results of duplicate analyses and N is the number of duplicates (SNEDECOR 1946, YOUDEN 1951)

For human plasma this figure was found to be ±2 93 in the range between 10 and 40 µg/100 ml plasma For rat plasma, the corresponding figure was ±1 43 in the same range

Determinations of 0 25 ml plasma samples versus 0 5 ml samples have also been performed The plasma concentrations were calculated and the differences between samples analyzed It was found that the differences between samples did not exceed those between duplicates

Accuracy

The accuracy of the method i e how nearly the values found, approach the "true" values, was tested by recovery experiments performed at different plasma corticosteroid levels, by adding 0 1 µg of hydrocortisone to 0 25 ml samples of human plasma and 0 05 µg of corticosterone to 0 25 ml samples of rat plasma

The mean value for hydrocortisone recovery from human plasma was found to be 94 5 ± 2 0% (standard error of the mean), and the mean value for corticosterone recovery from rat plasma 99 8 ± 3 6% (s e m)

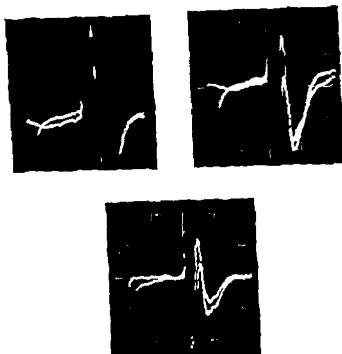


Fig 4 Fluorescence pattern at 475 mμ activating wavelength for corticosterone, hydrocortisone and plasma extracts
a) Corticosterone (higher) and hydrocortisone
b) Hydrocortisone (higher) and human plasma extract
c) Corticosterone (higher) and rat plasma extract

Sensitivity

As mentioned above, the standard deviation calculated from the difference between duplicate analyses of corticosterone in rat plasma was found to be 1 43 Assuming that this applies also to levels below 10 μg/100 ml, the smallest amount of corticosterone detectable by this method differing significantly from zero would be 2 86 μg/100 ml plasma at $p = 0.05$ in a 0 25 ml sample Under the same conditions, the corre sponding figure for hydrocortisone determination in human plasma would be 5 86 μg/100 ml Thus, the 0 25 ml samples used are sufficient for most purposes In determining the corticosterone level of hypophysectomized and adrenalectomized rats, however, 0 5 ml plasma samples are recommended

Specificity

It is possible to get a good picture of the specificity of the method by examining the localisation of fluorescence maxima from synthetic hydro-

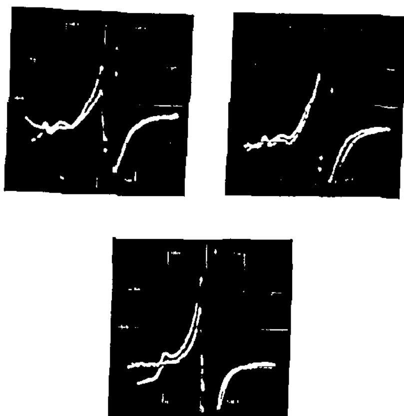


Fig 5 Fluorescence pattern at 550 mμ fluorescent wavelength for corticosterone, hydrocortisone and plasma extracts
a) Corticosterone (higher) and hydrocortisone
b) Hydrocortisone (higher) and human plasma extract
c) Corticosterone (higher) and rat plasma extract

cortisone and corticosterone standards compared with each other and with extracts from human and rat plasma This was done by connecting a cathode ray oscillograph with the spectrofluorometer and then taking photographs of the resultant curve made by the fluorescent point in passing over the screen With the spectrofluorometer used in this laboratory, the fluorescence peaks for synthetic hydrocortisone and corticosterone were found to be situated at 4750 Å activating wave length and 5500 fluorescent wave-length The test was first done with the emitted spectrum *moving along the abcissa, the activating wave-length fixed at 4750 Å* (fig 4 a–c), and then with the activating wave-length on the abscissa and *the fluorescent wave-length fixed at 5500 Å (fig 5 a–c)*

As can be seen from fig 4 a–c and 5 a–c, all curves have their peaks at the same activating and fluorescent wave-lengths Consequently no differentiation is possible between hydrocortisone and corticosterone by this method (fig 4 a and 5 a) As can be seen from fig 4 b–c and 5 b–c the

peaks for extracts from human and rat plasma are situated at the same wave lengths as the peaks for hydrocortisone and corticosterone standards, respectively This indicates the absence of interference by plasma factors other than ones with similar fluorescence peaks as corticosteroids Besides corticosteroids, the only organic compound in plasma known to give a high fluorescence at 4750/5500 Å is riboflavine However, this substance being insoluble in chloroform and methylene chloride, interference due to it can be excluded Other steroids besides hydrocortisone and corticosterone give in these circumstances only a trivial fluorescence and are found in plasma at extremely low concentrations (DE MOOR *et al* 1960) Thus it may be concluded, that hydrocortisone and corticosterone in plasma are almost exclusively measured by the method described

As mentioned above, no differentiation between these steroids can be achieved by the method It is important to recognise this for theoretical reasons, but in practice studies of corticoid levels in man and rat are predominantly concerned with only one of the steroids

Appendix· Steroid Levels under Certain Conditions.

Rat

a) Normal level The corticosteroid level in rat plasma was determined in relation to a corticosterone reference standard and expressed in terms of it

To obtain a ' resting level", a number of rats were rapidly decapitated in the animal room without anaesthesia Care was taken to disturb the animals as little as possible, as it has been shown that even small environmental changes produce adrenocortical stimulation (GUILLEMIN *et al* 1958, VAN DER VIES *et al* 1960) With the method used in this laboratory, using rats of our strain and weight range, the "resting level" of plasma-free corticosteroids has been determined to be 17 6 ± 1 9 µg per 100 ml plasma (s e m)

b) Hypophysectomy The plasma corticosteroid level was found to be 3 44 ± 0 37 µg 100 ml plasma (s e m) 24 hours after hypophysectomy

c) Adrenalectomy The plasma corticosteroid level was found to be 2 67 ± 0 35 µg 100 ml plasma (s e m) 24 hours after adrenalectomy

d) Influence of anaesthetic Repeated investigations have demonstrated that ether anaesthesia gives a more profound pituitary – adrenocortical response than pentobarbital (Mebumal NFN) SYDNOR & SAYERS 1954, MUNSON & BRIGGS 1955, HEDNER & RERUP 1960) According to this a group of animals was anaesthetized with pentobarbital sodium administered intraperitoneally as a 1 2% solution at a dose of 40 mg/kg After 10 minutes of complete anaesthesia the rats were decapitated, and

the plasma corticosteroid levels were estimated. Another group was anaesthetized by ether inhalation and decapitated after 10 minutes of complete anaesthesia. The results are shown in table 1.

Adrenalectomized and hypophysectomized rats were anaesthetized with ether. Their plasma corticosteroid levels did not indicate any adrenocortical stimulation.

As can be seen, determination of plasma corticosteroid level also shows ether anesthesia to effect a more powerful adrenocortical stimulation in rats than pentobarbital.

Table 1.
Influence of anaesthetic on corticosteroid concentration in rat plasma

Group	Anaesthetic	No of animals	Plasma cort ster level, µg/100 ml
1	None (contr)	12	17 6 ± 1 9
2	Pentobarbit	5	16 4 ± 2 5
3	Ether	6	41 4 ± 2 5

Test of significance	k	d f	p
Control versus pentob	0 38	15	>0 7
Control versus ether	7 45	16	<0 001
Pentobarb versus ether	6 90	9	<0 001

Man.

The corticosteroid level in human plasma has been determined relative to a hydrocortisone reference standard and expressed in terms of it

In samples taken at different times during the day, the "resting level" was found to be 18 1 ± 0 9 µg 100 ml plasma (n = 52) This is in agreement with values found by other investigators (SILBER *et al* 1958, DE MOOR *et al* 1960) Observations have indicated that adrenocortical function is characterised by a certain diurnal rhythm, first as changes in the amounts of 17-oxo-steroids excreted in the urine (PINCUS 1943), later as changes in the plasma corticosteroid level (BLISS, SANDBERG *et al* 1953, PERKOFF, EIK-NEES *et al.* 1959) These investigators drew blood samples from groups of persons at different times during the day and night This was not done in our investigation, only one sample being taken from each person. However, the time of each sampling was noted and it was observed that under these conditions the plasma corticosteroid levels was different at different times during the day. This day rhythm is shown in fig 6.

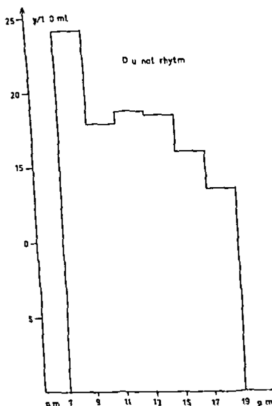


Fig 6 Day rhythm of human plasma cort costero d level

Summary

1 A fluorescence method for determining free corticosteroids in human and rat plasm has been investigated for its precision accuracy sensitivity and specificity

2 Levels of plasma corticosteroids in intact hypophysectomized and adrenalectomized rats are given

3 The influence of the anaesthetic (ether or pentobarbital) on plasma corticosteroid concentration in the rat is demonstrated

4 Resting levels and diurnal rhythms in human plasma corticosteroid levels are recorded

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Acta pharmacol et toxicol 1961, 18, 75–79

From the Biochemical Laboratory Northern General Hospital,
Edinburgh Scotland.

The Identification and Determination of some Hypotensive Drugs in Urine

By

S L. Tompsett, W Forshall, and D C Smith

(Received November 25 1960)

A number of substances have been introduced into clinical medicine for the treatment of hypertension These will be referred to below as hypotensive drugs The identification and determination of many of these substances in biological fluids, e g urine presents difficulties In general there is a lack of specific identification tests and many cannot be separated by extraction with organic solvents As a result it is difficult to effect concentration

It is believed that the procedure described in this paper represents some progress in this direction and although reference is only made to

(1) hexamethonium salts
(2) Bretylium tosylate (Darentin R)
(N (o-bromebenzyl)N ethyl N N dimethyl ammonium p-toluene sulphonic acid) and
(3) guanethidine (Ismelin R)
(2 perhydroazocin I'ylethylguanidium sulphate)

it is believed that it may have a more general application

In the text (2) and (3) will be referred to as bretylium and guanethidine, respectively it being assumed that examinations are being made of the basic radical only

Initially urine is applied to a cation exchange resin, the hypotensive drugs being retained The hypotensive drugs are eluted with 5 N hydro chloric acid the eluate being evaporated to dryness A solution of the residue is treated with ammonium reineckate, and the precipitated reineckates (including reineckates of the hypotensive drugs) are collected A solution of the reineckates in acetone is examined qualitatively and quantitatively by paper chromatography

Procedure.

Ion Exchange Chromatography

The column, containing 3 g of Dowex, 50 × 12 (height 70 mm, diameter 10 mm) was prepared as described in previous communications (TOMPSETT 1959, 1960)

To the column were applied 100 ml of urine + 10 ml of 10 N hydrochloric acid The column was washed with 100 ml of N hydrochloric acid and then eluted with 80 ml quantities of 2 5 N, 5 N and 8 N hydrochloric acid Eluates were combined as shown below

Hexamethonium	. 2 5 + 5 N acid eluates
Bretylium, Guanethidine	5 N + 8 N acid eluates

After dilution with water, these were evaporated to dryness in an all glass vacuum still

It has been found that a column of this size can cope adequately with 100 ml of urine, provided that one-tenth volume of 10 N hydrochloric acid is added, and examinations are limited to those substances requiring hydrochloric acid of a normality exceeding 1 to effect elution

Reineckate Precipitation

The residue was dissolved in 5 ml of water, which was transferred to a centrifuge tube To this were added 5 ml of a freshly prepared saturated solution of ammonium reineckate

At the same time, standards (0 5, 1 0, 2 0, 5 0 and 10 0 mg in 5 ml of water) of the substance under examination were set up

After standing at room temperature overnight, the tubes were centrifuged and the residues of reineckates were dissolved in acetone, e g 10 ml By colorimetric analysis of the acetone solutions, the amount of the substance present in the unknown may be calculated

Paper Chromatography

Paper Whatman no 1

Solvent System n-Butanol/Acetic Acid/Water (40 10/50)

Quantities of 20 μl of an acetone solution of the reineckate were applied to the paper Whenever possible, the concentration of the acetone solution was adjusted, e g by evaporation, so that 20 μl contained 80 μg of the base as determined by the colorimetric reineckate method The chromatogram was developed overnight in the solvent system above mentioned After development, the sheet was dried, and the position of the base was revealed by spraying with the Dragendorff reagent

Standards were set up at the same time, 20 μl of standard solutions (reineckate of the base in acetone) were applied The standards selected corresponded to 10 20 40, 60 and 80 μg of base/20 μl of acetone solution

Results and Discussion.

Various quantities of hexamethonium, bretylium, and guanethidine in 100 ml of N hydrochloric acid were applied to columns The columns were washed with 100 ml of N hydrochloric acid, and el[illegible]

Table 1

Hypotensive Drugs

Applied to a column of Dowex 50 × 12 (200 to 400 mesh)
Dimensions 70 × 10 mm
Weight 3 g
in 100 ml N hydrochloric acid

Recovery (%) in acid eluates (80 ml)

	N HCl	2 5 N HCl	5 N HCl	8 N HCl
Hexamethonium				
2 mg	1 4	8 6	80 2	1 6
5 mg	2 6	10 4	84 6	1 2
10 mg	1 8	14 6	79 2	1 6
Bretylium				
2 mg	0 9	1 1	64 7	30 2
5 mg	1 6	1 7	75 6	21 2
10 mg	1 2	2 1	73 8	21 8
Guanethidine (Ismelin)				
2 mg	1 3	1 8	62 3	26 2
5 mg	2 3	2 3	79 6	24 2
10 mg	3 1	2 7	70 3	34 2

Table 2

The recovery of *Hexamethonium Bretylium* and *Guanethidine* added to 100 ml of urine

	Quantity added (mg)	Recovery %
Hexamethonium		2 5 N + 5 N *hydrochloric acid Eluates*
	2	88 2
	5	105 6
	10	103 6
Bretylium		5 N + 8 N *hydrochloric acid Eluates*
	5	106 3
	8	101 0
	10	91 6
	20	94 8
Guanethidin		5 N + 8 N *hydrochloric acid Eluates*
	2	92 6
	5	94 8
	10	103 6

Determination as a solution of the reineckate in acetone

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Various quantities of hexamethonium, bretylium, and guanethidine in 100 ml of N hydrochloric acid were applied to columns The columns were washed with 100 ml of N hydrochloric acid, and elutions were then

sprayed with the Dragendorff reagent, whereas the 'normal' urinary constituents probably of dietary origin, do not react

The R_F values of the hypotensive drugs in the solvent system used are shown in table 3 The reineckate reagent travels to a spot of R_F about 0 84 but does not react with the Dragendorff reagent The reineckates of bretylium and guanethidine break up clearly during chromatography, the free bases travelling to their characteristic spots Hexamethonium reineckate is exceptional in this respect, and the compound does not break up unless the spot is treated with excess silver nitrate before chromatography If this is done, the insoluble silver reineckate remains at the origin, and the liberated hexamethonium base travels to its characteristic spot For this purpose, the dried spot is treated with excess 0 1 N silver nitrate before chromatography is begun The excess silver salt travels to approximately R_F 0 15 and with the Dragendorff reagent it gives an intense immediate reaction, which then fades completely

The urinary excretion of bretylium and guanethidine was studied after oral administration of various doses Final evaluation was made by the visual examination of paper chromatograms From the results shown in table 4 it will be noted that recovery by urinary excretion is low after oral intake

The method was devised for the qualitative and quantitative examination in urine of some hypotensive drugs in current clinical use Normal human urine contains substances that are retained by and eluted from the column under identical conditions and that also form insoluble reineckates, which may subsequently be dissolved in acetone These substances, however, do not react with the Dragendorff reagent on paper chromatograms and thus do not interfere Alkaloids when present will react similarly to the hypotensive drugs (TOMPSETT 1960), but some differentiation can be achieved by the use of solvent extraction

Summary.

A method is described for the qualitative and quantitative examination of some hypotensive drugs in human urine

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Table 3

The examination of *Hexamethonium Bretylium* and *Guanethidine* by paper chromatography

Paper Whatman No 1
Solvent System nButanol/Acetic Acid/Water
(40 / 10 / 50)

	R_F
Hexamethonium	0 11
Bretylium	0 77
Guanethidine	0 37

carried out with 80 ml quantities of 2 5 N, 5 N and 8 N hydrochloric acid respectively All fluids that had passed through the columns were collected and evaporated to dryness, and the drug content was determined colorimetrically by means of the reineckate method The results are shown in table 1 All drugs were retained by the columns Hexamethonium could be recovered quantitatively in the combined 2 5 N and 5 N hydrochloric acid eluates Bretylium and guanethidine could be recovered quantitatively in the combined 5 N and 8 N hydrochloric acid eluates

Various quantities of the drugs were then applied to columns in 100 ml of urine + 10 ml of 10 N hydrochloric acid The results shown in table 2 indicate that recoveries were quantitative Assessment was made by the colorimetric reineckate method

All human urines contain in variable quantities substances that react in a similar fashion to the hypotensive drugs in their reactivity towards the cation exchange resin and precipitation as reineckate On paper chromatograms, the hypotensive drugs produce a pink colour when

Table 4

The urinary excretion of *bretylium* and *guanethidin* after oral therapy

Dose (mg)	*Urinary Excretion**) (mg)	*Recovery %*
Bretylium		
4800	504	10 5
400	77	19 3
200	55	27 5
Guanethidine		
200	38	19 0
30	4	13 3

*) Within 24 hours after dosage

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Acta pharmacol et toxicol 1961, 18, 80-88

From the Biological Department, the Pharmaceutical Institute,
University of Oslo

Determination of Adrenaline on the Isolated Serotonin-Stimulated Rat Uterus

By

Kjell Briseid Jensen and Anne Marie Vennerod

(Received November 30 1960)

The inhibitory action of adrenaline on the isolated rat uterus is the basis of a sensitive and consequently also a relatively specific method for assaying the drug It was first described by DE JALON, BAYO & DE JALON (1945) and later modified by GADDUM, PEART & VOGT (1949) and GADDUM & LEMBECK (1949), who replaced acetylcholine as stimulating agent by the more stable carbachol and used (2 + 2) assays The statistical treatment of the results was by the method of BLISS (1944) and NOEL (1945) The assay method of GADDUM & LEMBECK (1949) was subsequently used by several other workers MUSCHOLL (1959) modified the procedure by using oxytocin instead of carbachol as agonist, thus making it possible to eliminate the effect of choline esters by the addition of atropine

In our work the method of GADDUM & LEMBECK (1949) was modified, essentially by omitting the numerous recovery doses of uninhibited agonist given during the assay and by increasing the number of adrenaline-inhibited contractions The error of assay was calculated as first described for isolated organs by SCHILD (1942) Serotonin was used as agonist This substance usually requires less load on the uterus to give linear log-concentration response curves than does acetylcholine, and notably less than oxytocin (BRISEID JENSEN & SUND 1960 a), it gives steeper log-dose (adrenaline) inhibition curves, and therefore more accurate assays

1. Technique.

A Uterus

Uteri from virgin rats weighing 150-180 g were suspended in de Jalon solution (GADDUM, PEART & VOGT 1949) usually maintained at 29° (± 0 1°) The rats were injected with stilboestrol (diethylstilboestrol) (10 µg/100 g) 18-24 hours before they

were killed The uterus was generally used soon after the animal s death, but was occasionally stored at +4° for not more than three days before use

B Agonist and Antagonist Solutions

Serotonin at concentrations of 8–20 ng/ml was used as agonist The range 10–14 ng/ml was most usual [illegible]

containing 10 mg ascorbic acid per litre The final concentrations of the adrenaline solutions were generally in the range 0 1–1 0 ng/ml, though occasionally still lower concentrations were used They were protected against light throughout the experimental period

C Apparatus and Dose Cycle

The experiments were done in an automatic assay apparatus from Casella Electronics Ltd, London The bath volume was about 3 ml, and the fluid was changed by emptying the vessel and refilling it from two 250 ml stock bottles containing de Jalon solution (wash fluid) and serotonin in de Jalon solution, and from four 100 ml stock bottles (light protected) containing the standard or test adrenaline solutions The apparatus being constructed for one antagonist and four agonists, for convenience the four antagonist stock bottles were connected to the apparatus by two three way stopcocks

The period of contact of agonist solution ranged from 20 seconds to 65 seconds, 30–50 seconds being most usual The washing consisted of a threefold change of wash fluid After the last washing the antagonist solution was left in contact with the muscle for about 2 minutes, the bath was then emptied and filled with agonist solution with no intervening washing The interval between doses was fixed at 4 minutes The bath was aerated

D Materials

Serotonin 5 hydroxytryptamine creatinine sulphate, Hoffmann-La Roche & Co Ltd Basel, Switzerland

[illegible] the adrenaline values refer to the base

E Procedure

Very thin or very thick muscles were not used The muscle was attached to an isotonic frontal writing lever (SCHILD 1947) yielding a [illegible]

[illegible] contractions, and generally the uterus became stable after 5 or 6 further contractions When this did not happen after 10–15 contractions, the load was slightly increased, but preferably to not more than 1 1 g

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Table 1

Calculation of adrenaline inhibition
(Assay 11, table 2)

Dose of serotonin 10 ng/ml
Standard adrenaline (S_1 and S_2) 0 25 and 0 50 ng/ml
Test - (U_1 and U_2) - - - -
Average recovery contraction before assay 74 mm
- - - after - 69 -
Sensitivity fall per contraction 0 18 mm (4 + 6 4 contractions)

Series	Doses of adrenaline	Contractions mm		Inhibition %
		Observed Inhibited	Calculated Non inhibited	
1	U_1	54	73 0	26
	S_2	45	73 0	38
	U_2	43	73 0	41
	S_1	56	72 5	23
2	U_2	40	72 5	45
	U_1	52	72 5	28
	S_1	52	72 0	28
	S_2	41	72 0	43
3	U_2	39	72 0	46
	S_1	53	71 5	26
	U_1	53	71 5	26
	S_2	37	71 5	48
4	U_2	38	71 0	46
	U_1	51	71 0	28
	S_1	51	71 0	28
	S_2	35	70 5	50
5	U_1	50	70 5	29
	U_2	38	70 5	46
	S_2	37	70 0	47
	S_1	48	70 0	31
6	U_2	38	70 0	46
	U_1	49	69 5	29
	S_1	50	69 5	28
	S_2	36	69 0	48

2 Comments on the Technique.

A Agonist

A number of experiments were carried out with oxytocin (syntocinon ®, Sandoz) as agonist From the theoretical aspect this substance offers some advantages in adrenaline assays when other compounds acting on the uterus could be present As already mentioned, MUSCHOLL (1959) used oxytocin, adding atropine at the same time, to determine adrenaline in presence of acetylcholine Of more general interest is the fact that no

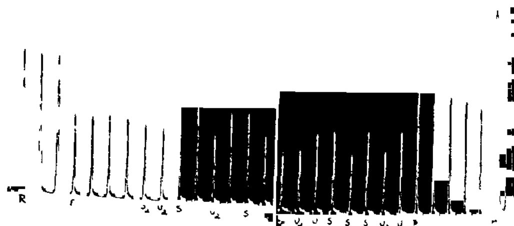


Fig. 1. Typical adrenaline assay.
The 2 middle series of a complete number of 6 are omitted from the record.
Serotonin 6 ng/ml bath was used as agonist.

R = *Recovery doses of serotonin.*
E = Equilibration doses. 0.2 ng/ml of adrenaline added.
S_1 and S_2 = Standard adrenaline. 0.1 and 0.2 ng/ml.
U_1 and U_2 = Unknown adrenaline. 0.1 and 0.2 ng/ml.
M = Maximum doses of serotonin. 400 and 800 ng/ml.

As soon as the uterus was stable, standard adrenaline was administered (usually 0.25 ng/ml); after a transitory stronger inhibition the heights of contraction levelled out at a higher value. At this point doses of unknown adrenaline solution were put into the bath to obtain a rough estimate of the adrenaline content. 3 recovery doses of serotonin (for purposes of calculation) were then given, as well as 4 or 5 doses of standard adrenaline in order to restore a stable level of inhibition. The assay was accordingly begun as the usual (2 + 2) assay with a dose ratio of 2 and 6 series of *four randomized doses. After the end of the assay 3 recovery doses of serotonin were* given. The maximum contractions were obtained by giving two doses of serotonin about 50 times higher than used in the assay. Fig. 1 shows an example of an assay from the moment on when the preliminary recovery doses of serotonin were given. The 2 middle series are omitted from the tracing.

F. Calculation of Inhibition

The change in uterine sensitivity during the experimental period, usually a fall, was calculated on the basis of the average contraction values of 3 recovery doses of serotonin given at the beginning and at the end of the (2 + 2) assay. The figures thus obtained made it possible to calculate a theoretical recovery contraction height corresponding to every observed adrenaline inhibited contraction. The individual contractions were *measured to the nearest mm* and the *inhibition percentages* were found from the theoretical recovery contraction heights, which were calculated to the nearest 0.5 mm, as is shown in table 1. The inhibition percentages are given as whole numbers.

finished We therefore decided to discard in advance such thin muscles to save time It was seldom that muscles from stilboestrol injected rats had to be discarded for this reason

Spontaneous contractions were unusual under the conditions employed and were always quickly abolished

A load on the uterus of 0 8–0 9 g occasionally gave no stability, and the load was then increased If more than about 1 1 g was necessary, the dose response curve became less steep and the error of assay larger In that event (for frequency, see Results) a new uterus preparation was suspended in the bath without delay, so as to avoid the need to discard the muscle during the assay

Table 3

Dose and load data from the 10 adrenaline assays summar sed in table 2.

Assay	Load in g	Serotonin		Adrenaline ng/ml	
		ng/ml	contact time in sec	Standard doses	Test doses
1	1 0	20	20	0 50–1 00	0 60–1 20
2	0 8	10	35	0 50–1 00	0 50–1 00
3	0 8	10	40	0 20–0 40	0 15–0 30
4	0 8	12	44	0 50–1 00	0 63 1 26
5	0 8	8	60	0 30–0 60	0 25–0 50
6	0 9	14	65	0 08–0 16	0 09–0 18
7	0 8	10	50	0 38–0 76	0 38–0 76
9	0 8	10	32	0 50–1 00	0 47–0 94
10	0 8	10	33	0 25–0 50	0 26–0 52
11	0 9	10	33	0 25–0 50	0 25–0 50

A correctly adjusted time of contact for the agonist was essential If it was too short the longer contractions were "cut", if it was too long the muscle often contracted twice and became unstable

For most uteri one or two of the adrenaline inhibited doses of serotonin after stimulation with pure serotonin were not reduced down to the inhibition level obtainable This fact explains the use of some standard adrenaline doses after the three serotonin recovery doses before assay

In GADDUM & LEMBECK's (1949) method two recovery doses after each adrenaline inhibited dose were intended to bring the uterus up to, or nearly up to, a stable recovery level, thus delaying the fall in the sensitivity of the muscle In our experience these numerous recovery doses are not necessary The results indicate that the proposed change between different adrenaline inhibited contraction levels gives a considerable uniformity of contractions at each of these levels, and the fall in sensitivity is not

specific antagonist seems to have been found so far for this polypeptide (BRISEID JENSEN & SUND 1960 a), which much increases the specificity of adrenaline determinations based on oxytocin. However, in our hands the polypeptide did not for practical reasons prove successful as a stimulating agent. Several assays had to be discarded because of poor precision. The confidence limits ($P = 0.05$) often far exceeded the range 83–120%, which covered all the experiments done with serotonin as agonist (see table 2). According to previous investigations (BRISEID JENSEN & SUND 1960 b) the adrenaline inhibitions of, for example, acetylcholine, oxytocin and serotonin are fundamentally the same, and similar contractile activities of any of these substances should under the same conditions be influenced to the same degree by variations in adrenaline concentrations. However, as the various agonists required markedly different loads on the muscle, the steepness of the log concentration curves obtainable varied considerably and also the steepness of the log-dose (adrenaline) inhibition curves varied accordingly. Whereas oxytocin required a high load and often an adrenaline dose ratio of 3,4, sometimes even more, serotonin invariably differentiated well with a dose ratio of 2.

Table 2

Accuracy and precision of the method

Different adrenaline solutions (10) assayed on uteri from different rats. Further details from the assays are given in tables 3 and 4.
s/b: Ratio of the standard deviation to the slope of the regression line.

Assay	Adrenaline µg/ml		Actual error as % of true potency	s/b	Fiducial limits* ($P = 0.05$)
	Theoretical	Observed			
1	150.0	156.0	+4.0	0.036	93–108
2	100.0	95.0	5.0	0.073	86–116
3	50.0	49.8	0.4	0.065	87–115
4	125.0	122.0	2.4	0.050	90–111
5	154.0	157.0	+1.9	0.081	84–118
6	182.0	191.0	+4.9	0.059	89–113
7	50.0	50.6	+1.2	0.034	93–107
8			Discarded during assay, see text		
9	23.5	24.9	+6.0	0.058	89–112
10	37.5	36.4	2.9	0.093	83–120
11	41.7	41.4	−0.7	0.032	94–107

B. Procedure

Thin muscles sometimes lost sensitivity quickly. An even fall in contraction height should not affect the results, since the proposed calculation procedure takes this possibility into account. However, the sensitivity of such uteri sometimes declined so rapidly that a (2 + 2) assay could not be

even after some weighting, the third muscle did not differentiate sufficiently

The statistical procedure described by SCHILD (1942) was used for the treatment of results. Table 2 shows that the accuracy as well as the precision of the method was satisfactory. The average departure from the true adrenaline content was 3% (range −5.0 to +6.0%) and the range of fiducial limits observed was 94–107% to 83–120% ($P = 0.05$). The mean value for the ratio of standard deviation of a single response to the slope of the regression line (s/b) was 0.058 (range 0.032 to 0.093).

The standard and test curves were parallel ($P > 0.05$) in all assays. The use of (2 + 2) assays eliminates the possibility of testing linearity in the individual experiments. Certainly the tests of parallelism give some security against serious departures from linearity if the chosen dose ratios between standard and test are not too near to 1. However, as experience shows that the region of linearity varies from one muscle preparation to another, it seems safer to attach importance to a good preliminary guess of strength of unknown adrenaline solution and the use of a standard to test dose ratio as near to 1 as possible. From table 3 it can be calculated that the ratios ranged between 0.8 and 1.3.

In most of the assays the uteri quickly became stable, and an *increase* of load was unnecessary. The duration of an assay was usually 4–5 hours from the moment the uterus was suspended in the bath. About an hour and a half was spent in relaxing and stabilising on a submaximal dose of serotonin, thus giving a true experimental time of about 3 hours.

Some additional data from the assays are given in tables 3 and 4.

Summary.

The rat uterus method for adrenaline assay proposed by DE JALON, BAYO & DE JALON (1945), and further developed by GADDUM & LEMBECK (1949), has been modified. *Serotonin was used* as agonist, the recovery doses of uninhibited agonist during assay omitted and the *error* of assay calculated as described by SCHILD (1942). The assay were performed as (2 + 2) assays with a dose ratio of 2 and 6 series of 4 doses.

Serotonin was used at concentrations of 8–20 ng/ml and adrenaline most often in the range 0.1–1.0 ng/ml.

To test the accuracy and precision of the method, 10 different, unknown adrenaline solutions were assayed. The average departure from the true adrenaline content was 3% (range −5.0 to +6.0%) and the range of fiducial limits ($P = 0.05$) was 94–107% to 83–120%.

The mean value for the ratio of standard deviation to slope of regression line (s/b) was 0.058 (range 0.032–0.093).

Table 4.

Contractions in the 10 adrenaline assays summarised in table 2

Assay	Level in mm		Change ±mm	Maximum in mm	Highest		Lowest	
	Before assay	After assay			mm	% of max	mm	% of max
1	66	59	−7	198	49	25	28	14
2	90	54	−36	130	86	66	29	22
3	86	85	−1	111	75	68	44	40
4	88	94	+6	113	72	64	45	40
5	60	44	−16	87	39	45	10	11
6	66	79	+13	135	63	47	29	21
7	91	87	−4	114	67	59	39	34
9	79	74	−5	108	61	56	33	31
10	95	95	0	106	88	83	63	59
11	74	69	−5	97	56	58	35	36

pronounced In fact the sensitivity of the uteri was unchanged or even increased during assay in some experiments (table 4) This may partly be due to the fact that serotonin requires only a small load to give stable contractions and partly to the reduction of experimental time as a result of omitting the recovery doses It seemed probable that the proposed change between different adrenaline doses, without any recovery doses in between, would tend to flatten the log-dose (adrenaline) inhibition curves, every adrenaline dose being influenced by the preceding dose However, comparative experiments on the same uterus with and without recovery doses showed no significant differences in steepness

3. Results.

To test the accuracy and precision of the described method 10 different adrenaline solutions were assayed Because of the variations between uteri, muscles from different rats were used in the experiments The adrenaline contents of the test solutions were unknown to the observer. Fourteen uterus preparations were suspended in the bath, 3 of them were discarded early and 1 was discarded during the assay to obtain the results of the 10 unknown adrenaline solutions The results are given in table 2

In experiment number 8 a (2 + 2) assay was begun in spite of considerable instability, which in part persisted when the load was increased from 0 8 to 1 0 g After a while the sensitivity of the muscle decreased rapidly, and the assay was stopped Three uteri were discarded at a preliminary stage of the experiments before assays were started, and the results of the experiments are accordingly omitted from table 2 For two of these muscles the discarding was due to a considerable lack of stability,

even after some weighting, the third muscle did not differentiate sufficiently

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The standard and test curves were parallel (P > 0 05) in all assays The use of (2 + 2) assays eliminates the possibility of testing linearity in the individual experiments Certainly the tests of parallelism give some security against serious departures from linearity if the chosen dose ratios between standard and test are not too near to 1 However, as experience shows that the region of linearity varies from one muscle preparation to another, it seems safer to attach importance to a good *preliminary guess of* strength of unknown adrenaline solution and the use of a standard to test dose ratio as near to 1 as possible From table 3 it can be calculated that the ratios ranged between 0 8 and 1 3

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Some additional data from the assays are given in tables 3 and 4

Summary.

The rat uterus method for adrenaline assay proposed by [illegible]

[illegible] assay omitted and the error of assay calculated as described by *SCHILD (1942)* The assay were performed as (2 + 2) assays with a *dose ratio* of 2 and 6 series of *4 doses*

Serotonin was used at concentrations of 8–20 ng/ml and adrenaline most often in the range 0 1–1 0 *ng/ml*

To test the accuracy and precision of the method, 10 different, unknown adrenaline solutions were assayed The average departure from the true adrenaline content was 3% (*range* −5 0 to +6 0%) and the range of fiducial limits (P = 0 05) was 94–107% to 83–120%

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4	88	94	+6	113	72	64	45	40
5	60	44	−16	87	39	45	10	11
6	66	79	+13	135	63	47	29	21
7	91	87	−4	114	67	59	39	34
9	79	74	−5	108	61	56	33	31
10	95	95	0	100	88	83	63	59
11	74	69	−5	97	56	58	35	36

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In experiment number 8 a (2 + 2) assay was begun in spite of considerable instability, which in part persisted when the load was increased from 0.8 to 1.0 g. After a while the sensitivity of the muscle decreased rapidly, and the assay was stopped. Three uteri were discarded at a preliminary stage of the experiments before assays were started, and the results of the experiments are accordingly omitted from table 2. For two of these muscles the discarding was due to a considerable lack of stability,

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From the Biological Department, the Pharmaceutical Institute,
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Biological Assay of Adrenaline in the Presence of Local Anaesthetics

By

Kjell Briseid Jensen and Anne Marie Venneröd

(Received November 30 1960)

It is not possible to distinguish between (−)- and (+)-adrenaline by chemical tests As (−) adrenaline is normally used for injections, and as racemization during storage cannot be excluded with certainty, (Morch 1958) a biological assay method is necessary for adequate control of such solutions The determination of adrenaline in presence of local anaesthetics presents a special problem, as the latter can affect the biological methods Few investigations on the biological determination of adrenaline in the presence of local anaesthetics seem to have been conducted Using the rat blood pressure Morch (1960) found that all the anaesthetics examined affected the results to some degree However, an addition to the standard solutions of the same quantity of the local anaesthetic as was present in the test solutions rendered possible the determination of adrenaline The same procedure was used for noradrenaline determinations

In our work we have tried to carry out the assays on the isolated, serotonin stimulated rat uterus (Briseid Jensen & Venneröd 1961) by direct dilution of the adrenaline – local anaesthetic solution and without addition of local anaesthetic to the standard solution Such a procedure seemed reasonable, as the rat uterus method is highly sensitive compared with the rat blood pressure method, 0 1–1 0 ng/ml of adrenaline being required in the former, as against 1–5 µg/ml in the latter Besides the local anaesthetics usually employed along with adrenaline, namely procaine, lidocaine, and carbocain, we also examined cincaine and tetracaine

1. Technique.

Both technique and statistical treatment of the results were as previously described for pure adrenaline solutions (Briseid Jensen & Venneröd 1961)

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local anaesthetic in question at increasing concentrations Each concentration was used several times to make possible the detection of any slowly developing effect The working conditions in these experiments were as stated in the description of method (BRISEID JENSEN & VENNERÖD 1961). The results are given in table 1 From knowledge of the adrenaline concentrations necessary for assays and the relative concentrations of adrenaline and local anaesthetics most usually employed (*vide infra:* Comments on the Results), the results in table 1 seemed to call for further investigations of combinations of adrenaline with lidocaine, procaine or carbocain

Table 2

Determination of adrenaline in presence of procaine or lidocaine

Theoretical and observed concentrations

Concentration of local anaesthetic 20 mg/ml
s/b Ratio of standard deviation to slope of regression line Further details of the assays in tables 3 and 4

Assay	Adrenaline µg/ml		Actual error as % of true potency	s/b	Fiducial limits % (*P* = 0 05)
	Theoretical	Observed			
Procaine 1	41 1	40 8	0 7	0 054	89–112
– 2	24 6	24 0	−2 4	0 048	91–110
– 3	45 1	43 4	−3 8	0 043	91–109
– 4	49 3	50 4	+2 2	0 024	95–105
– 5	41 1	40 6	−1 2	0 054	90–112
– 6	20 0	19 3	−3 6	0 049	91–110
– 7	23 0	23 4	+1 7	0 045	91–109
Lidocaine 1	45 2	44 9	−0 7	0 043	92–109
2	31 4	31 1	−1 0	0 043	92–109
– 3	29 5	29 2	−1 0	0 040	92–108
– 4	49 1	48 4	−1 4	0 044	91–109
5	32 7	32 4	−0 9	0 033	94–107
– 6	5 00	4 98	−0 4	0 050	90–111
– 7	5 00	4 66	−6 8	0 051	90–111
– 8	4 00	3 90	−2 5	0 026	95–107

Table 2 shows the results of determinations of adrenaline in the presence of procaine or lidocaine

In all the experiments a 2% concentration of local anaesthetic was used, and the corresponding adrenaline concentrations were chosen from the range normally employed for injection

In the procaine experiments the average departure from the true adrenaline content was 2 2% (range −3 8 to +2 2%), and the range of fiducial limits observed was from 95–105% to 89–112% The mean value for the ratio of the standard deviation to the slope of the regression line (s/b) was 0 045 (range 0 024 to 0 054) In the lidocaine experiments the

Besides the chemicals mentioned in that paper, the substances used were
Carbocain Carbocain hydrochlor , AB Bofors-Nobelkrut, Karlskoga, Sweden
Cincaine Nupercain ® (chlorhydrat), Ciba, A G Basel, Switzerland
Lidocaine Xylocain ® (hydrochlor), Norsk Astra A/S Fjellhamar, Norway
Procaine Procain hydrochlor , Farbwerke Hoechst, A G Frankfurt a M Germany
Tetracaine Tetracain hydrochlor , Farbwerke Hoechst A G Frankfurt a M Germany
(+)-*Adrenaline* (+) Adrenaline, L Light & Co Ltd Colnbrook, England
(+) *Adrenaline* (+) Adrenaline, California Corporation For Biochemical Research, Los Angeles, U S A

The concentrations of the local anaesthetics mentioned in this paper refer to the chlorides, the adrenaline values refer to the base, the doses of serotonin to 5 hydroxy tryptamine creatinine sulphate All percentages are w/v

The local anaesthetics used were dissolved in the adrenaline solutions immediately before the beginning of the experiments

2. Results.

The two preparations of (+) adrenaline were assayed against (−) adrenaline to estimate the relative inhibitory effects on the rat uterus The Light & Co preparation seemed to be 40–50 times less active than (−)-adrenaline, the preparation from the California Corporation For Biochemical Research 60–70 times less active

Table 1

Effects of various local anaesthetics on the isolated serotonin stimulated rat uterus

μg/ml bath	Local anaesthetic: Lidocaine	Procaine	Carbocain	Tetracaine	Cincaine
0 1	0	0	0	0	0
1 0	0	0	0	Strongly depressed	Strongly depressed
3 0	0	0	0	–	–
10 0	0	Depressed	Unstable	–	–
30 0	Unstable weakly depressed	–	Unstable weakly depressed		–
100 0	Depressed	–	Unstable strongly depressed	–	–

Preliminary experiments were conducted to determine at what concentrations the various local anaesthetics would influence contractions of the rat uterus stimulated by serotonin This was done by stabilizing the muscle on a submaximal dose of serotonin and then testing as antagonist the

Table 3 gives the serotonin doses, the loads on the uteri and the concentrations of adrenaline and local anaesthetic used in the experiments mentioned in table 2 Table 4 shows the figures for contraction The adrenaline contents of the test solutions were always unknown to the observer

3. Comments on the Results.

The local anaesthetics examined were those most frequently used along with adrenaline in injections procaine, lidocaine and carbocain The highest concentrations of the substances normally used in injections are 2%, and the corresponding range of adrenaline contents 0 0005–0 005% (5 50 μg per ml) In the solutions prepared for assays we accordingly fixed the concentration of local anaesthetic at 2% and varied the adrenaline contents within the limits mentioned above This means that the local anaesthetic was present in amounts 400–4,000 times larger on a weight basis As the concentrations of adrenaline necessary for inhibiting the rat uterus usually fall in the range 0 1–1 0 ng/ml (BRISEID JENSEN & VENNEROD 1961) the concentration of local anaesthetic in the final solution varies between 0 15 and 1 5 μg/ml for an uterus of average sensitivity

From table 1 it can be seen that both tetracaine and cincaine strongly affect the rat uterus at concentrations of 1 μg/ml Accordingly an assay method for adrenaline based on a comparison of a simple dilution of the test fluid with a standard solution free from local anaesthetic is out of the question With the other local anaesthetics examined the results of the preliminary experiments did not exclude the possibility of such an assay procedure and further investigations were begun

Table 2 gives the results of the procaine adrenaline and the lidocaine-adrenaline assays The lowest adrenaline concentrations given in the table were the lowest concentrations that could safely be determined without significant interference from the local anaesthetic

The determinations of adrenaline in presence of lidocaine were satisfactory over the whole range of adrenaline concentrations examined Accordingly all the results observed are shown in table 2 It can be concluded that adrenaline may be successfully determined in solutions containing quantities of lidocaine up to 4,000 times larger on a weight basis than those of adrenaline

When an attempt was made to assay adrenaline in the presence of procaine, it seemed difficult to obtain good results with amounts of the local anaesthetic about 4,000 times higher than that of adrenaline At such concentration ratios the muscle preparations tended to become unstable, being sometimes stimulated and sometimes depressed Quantities of procaine about 1,000 times larger than those of adrenaline,

Table 3.

Dose and load values from the adrenaline assays summarised in table 2

Assay	Load in g	Serotonin		Adrenaline ng/ml		Local anaesthetic µg/ml
		ng/ml	contact time in sec	Standard doses	Test doses	
Procaine 1	1 1	8	35	0 50–1 00	0 46–0 92	0 22–0 44
– 2	1 0	8	50	0 19–0 38	0 19–0 38	0 15–0 30
– 3	0 9	8	–	0 25–0 50	0 23–0 46	0 10–0 20
– 4	0 9	16	30	0 25–0 50	0 25–0 50	0 10–0 20
– 5	0 9	6	30	0 11–0 22	0 10–0 20	0 05–0 10
– 6	0 9	20	40	0 10–0 20	0 10–0 20	0 10–0 20
– 7	1 1	14	45	0 25–0 50	0 23–0 46	0 20–0 40
Lidocaine 1	0 9	10	35	0 25–0 50	0 23–0 46	0 10–0 20
– 2	0 9	6	42	0 38–0 76	0 38–0 76	0 24–0 48
– 3	0 9	14	35	0 19–0 38	0 18–0 36	0 12–0 24
– 4	0 9	8	30	0 25–0 50	0 25–0 50	0 10–0 20
– 5	0 9	5	45	0 13–0 26	0 12–0 24	0 08–0 16
– 6	0 8	6	45	0 25–0 50	0 25–0 50	1 00–2 00
– 7	0 9	20	30	0 13–0 26	0 13–0 26	0 50–1 00
– 8	0 9	10	50	0 19–0 38	0 20–0 40	1 00–2 00

Table 4.

Contraction values from adrenaline assays summarised in table 2

Assay	Level in mm		Change ± mm	Maximum in mm	Highest		Lowest	
	Before assay	After assay			mm	% of max	mm	% of max
Procaine 1	76	66	−10	–	–	–	–	–
– 2	77	73	− 4	128	54	42	30	23
– 3	79	57	−22	122	57	47	18	15
– 4	70	74	+ 4	112	57	51	35	31
– 5	67	74	+ 7	140	67	48	33	24
– 6	60	69	+ 9	107	52	49	36	34
– 7	84	74	−10	134	53	40	38	28
Lidocaine 1	64	54	−10	91	42	46	24	26
– 2	77	63	−14	102	55	54	28	27
– 3	76	59	−17	102	46	45	29	28
– 4	61	59	− 2	94	43	46	29	31
– 5	79	71	− 8	129	54	42	28	22
– 6	72	56	−16	104	54	52	31	30
– 7	63	57	− 6	93	49	53	14	15
– 8	62	51	−11	91	43	47	28	31

average departure from the true adrenaline content was 2 0% (range −6 8 to −0 4%, and the range of fiducial limits was from 95–107% to 90–111%. The mean value for s/b was 0 041 (range 0 026 to 0 051).

The standard- and test-curves were parallel in all assays ($P > 0.05$)

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From the Department of Pharmacology University of Helsinki
(Professor Armas Vartiainen M D)

Effect of Pyrocatechol on the Change of Noradrenaline Content Induced in Rat Brain by Raunescine*

By

A J Jäättelä and M K Paasonen

(Received January 12, 1961)

BACQ demonstrated in 1936 that pyrogallol and other catechols increase the duration of response to adrenaline and sympathetic stimulation *in vivo* More recent work has indicated that O methylation by catechol O-methyltransferase enzyme is the principal pathway for the metabolism of adrenaline and noradrenaline (cf AXELROD 1959 and ARMSTRONG & MCMILLAN 1959) Other orthodiphenols are also methylated by this enzyme Catechol (BACQ 1959) and pyrogallol (AXELROD & LAROCHE *1959)* both inhibit O methyltransferase, and a logical explanation for this is that they act by competitive inhibition

Raunescine is a Rauwolfia alkaloid chemically and pharmacologically related to reserpine (PAASONEN & DEWS 1958, KARKI & PAASONEN 1959) 5-Hydroxytryptamine (5 HT) is mainly metabolised by *monoamine* oxidase, when rats were treated before raunescine with an inhibitor of this enzyme, iproniazid, there was a transient increase, instead of a decrease, in the 5 HT content of brain tissue (PAASONEN & KARKI 1958) No increase was seen in noradrenaline content.

Our work has been undertaken mainly to find out whether the response of [illegible] uld [illegible] as [illegible] effect was also studied on the toxicity of adrenaline in mice and on the eyelid ptosis induced in rats by reserpine

Methods.

Noradrenaline in the Rat Brain

Male Sprague-Dawley rats [illegible]
done at room temperature [illegible]
brain excluding the cerebe[illegible]

* Supported by grants from the Sigrid Jusélius Foundation and State Commission for Natural Sciences.

however, did not interfere significantly with the assays, as is evident from table 2 This concentration ratio includes a considerable number of the procaine-adrenaline combinations used in injections

The adrenaline concentrations used in injections along with carbocain are generally lower than those used with the above mentioned local anaesthetics We accordingly chose adrenaline quantities as low as 5 μg/ml (or 4,000 times lower than the amounts of carbocain) for all the experiments Some of the assays were satisfactory both in accuracy and limits of error, but in other assays the uteri became stimulated by the local anaesthetic and the adrenaline amounts consequently appeared too low Further, some assays had to be stopped because of instability of the muscle preparations It must be concluded that adrenaline cannot be properly determined with the rather unfavourable concentration ratio of adrenaline to carbocain chosen Samples with higher concentrations of adrenaline were not examined, but by analogy with the procaine experiments, might well give better assay results

Summary.

An assay method for adrenaline based on the inhibition of the serotonin stimulated rat uterus (BRISEID JENSEN & VENNEROD 1961) has been used for determining adrenaline in the presence of local anaesthetics The assays were carried out by direct dilution of the adrenaline local anaesthetic test solutions, and without addition of local anaesthetic to the adrenaline standard solutions

The results show that adrenaline could be assayed in the presence of 4,000 *times larger amounts (by weight) of lidocaine* This corresponds to the most extreme concentration ratio of the substances normally used in injections

Adrenaline could also be satisfactorily assayed in the presence of 1,000 times larger amounts of procaine, a concentration ratio that includes a considerable number of the adrenaline procaine combinations used for injection When a ratio of 4,000 was tried, the uterine preparations became unstable, being weakly depressed or weakly stimulated

When an attempt was made to assay adrenaline in the presence of 4 000 times larger amounts of carbocain, some of the assays were satisfactory and some were unsuccessful Thus a valid adrenaline assay was not possible As the adrenaline concentrations used for injection along with carbocain are generally lower than those used with lidocaine or procaine, a higher adrenaline carbocain ratio was not examined

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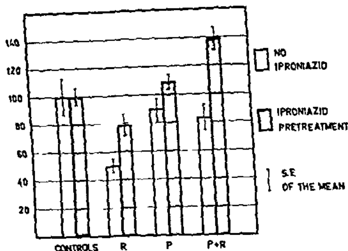


Fig 1 Effect of raunescine (R) and pyrocatechol (P) with and without iproniazid pretreatment on the brain noradrenaline content The results are given as percentages of the corresponding control groups For further explanation see text

amount of amine in the brains of rats also treated with iproniazid and pyrocatechol

Reserpine Eyelid Ptosis

Three hours after the reserpine injection the eyes were completely closed Pyrocatechol up to a dose of 50 mg/kg had no effect on this reaction When 100 mg/kg of iproniazid were injected 30 minutes after the reserpine the development of ptosis was not prevented In these animals no response was seen after 10 mg/kg of pyrocatechol, but 25 mg/kg of this opened the eyes in about 15 minutes, and they remained open for 10 to 15 minutes After 50 mg/kg of pyrocatechol the ptosis was abolished for about an hour

Even at a dose of 100 mg/kg resorcinol and hydroquinone were inactive when injected into rats pretreated with reserpine and iproniazid

When the closure of eyes was induced by chloralose, combined treatment with iproniazid and pyrocatechol at the maximum doses mentioned above produced no opening of the eyelids

Symptoms observed

In rats pyrocatechol within 2 to 3 minutes caused, tremor lasting from 10 to 15 minutes Occasionally there were convulsions, especially when doses of 50 mg/kg or higher were employed Slight pilo erection was also

subsequent procedure was the same as described by PAASONEN & KRAYER (1958) and essentially the same as suggested by von EULER (1956) The samples were analysed by the cat blood-pressure method Before beginning the assay a combination of drugs as suggested by KARKI (1956), was administered

Raunescine (S B Penick & Co , New York) was dissolved in glacial acetic acid and the solution was diluted with water so that the final concentration of acetic acid was not more than 2% The dose of raunescine was 5 mg (2 ml)/kg and was given intra peritoneally The animals were killed two hours after the raunescine injection *Pyrocatechol* (E Merck A G , Darmstadt) at a dose of 25 mg/kg was administered subcutaneously 6 times at ½-hour intervals, the first injection being given an hour before the raunescine *Iproniazid* phosphate (F Hoffmann La Roche & Co , Basel) was injected subcutaneously at a dose of 100 mg/kg of the base on the previous evening about 16 hours before the raunescine All rats were kept without food after iproniazid administration The control rats received injections of the corresponding solvents which was saline except for raunescine Student's t-test was used to test the significance of the difference of two means

Reserpine eyelid Ptosis

To induce the eyelid ptosis, 1 5 mg/kg of *reserpine* (reserpine amp , Lääke Oy, Turku) was injected intraperitoneally into rats, the controls receiving the solvent alone The time and degree of palpebral fissure reaction was estimated by inspection, and at least four rats were used at each dose level studied In these experiments, besides iproniazid and pyrocatechol *resorcinol* (E Merck A G Darmstadt) *hydroquinone* (E Merck, A G , Darmstadt) and *chloralose* (British Drug House Ltd , London) were used All these drugs were injected subcutaneously The design of experiments and the doses are given below

Toxicity of Adrenaline

Adrenaline (Rhone – Poulenc, Paris) was dissolved in hydrochloric acid and the solution was then diluted with saline a dose of 1 5 mg/kg was injected subcutaneously at half hourly intervals Half of the animals received also 20 mg/kg of pyrocatechol subcutaneously, but the injection was given as far as possible from the site of the adrenaline injection The first pyrocatechol dose was given half an hour before the adrenaline, and administration of both substances was continued until death of the animal

Results,

Noradrenaline in the Brain

Pyrocatechol alone at the dosage used had no clear effect on the brain noradrenaline content When given with raunescine, however, pyrocatechol treatment had an inhibitory action on the noradrenaline depletion (fig 1). The amine content in the group receiving raunescine only was 0 147 ± 0 015 (S E of the mean) μg/g and in the group treated with pyrocatechol and raunescine 0 244 ± 0 026 μg/g These two means are significantly ($p < 0\ 01$) different

Iproniazid treatment inhibited noradrenaline depletion induced by raunescine, as was already known (PAASONEN & KARKI 1959) Pyrocate-

protection of noradrenaline released from sympathetic nerve endings The role of catecholamines is further emphasized by the finding of activity in only those dihydroxybenzenes capable of inhibiting O-methyltransferase Further, the pyrocatechol iproniazid treatment does not inhibit the eyelid ptosis induced by an ordinary hypnotic agent, chloralose, which is not known to release noradrenaline from tissues

Iproniazid increases the toxicity of adrenaline and noradrenaline only in special circumstances (Borowitz & North 1959) When large amounts are injected there may be little opportunity to inactivate any of the dose given before a lethal level is reached Consequently the lack of clear potentiation of adrenaline toxicity to mice by pyrocatechol is not surprising

The general symptoms produced in rats by pyrocatechol with or without raunescine were particularly marked when iproniazid was also administered The overall picture was that of no clear motor stimulation but rather raised irritability, including tremor and convulsions

The results indicate that both oxidative deamination and O methylation are metabolic pathways for endogenous noradrenaline in the nervous tissue of rats It cannot be decided whether one of these mechanisms is more important than the other

Summary

1 Pyrocatechol pretreatment inhibits the noradrenaline depleting action of the Rauwolfia alkaloid raunescine, in the rat brain When iproniazid is also given raunescine produces an increase in the brain noradrenaline

2 Pyrocatechol and iproniazid counteract the reserpine induced eyelid ptosis only when both substances are injected into the rat Meta- and paradihydroxybenzenes (resorcinol and hydroquinone) in place of pyrocatechol are inactive

3 Pyrocatechol does not clearly increase the toxicity of adrenaline repeatedly given into mice

The results indicate that both monoamine oxidase and catechol O methyltransferase can inactivate endogenous noradrenaline in the nervous tissue of rats

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noted, but no exophthalmos The animals remained at one place in the cage and were over irritable to touch All the symptoms were intensified when iproniazid had been administered to the animals on the previous evening Raunescine treatment also agravated the symptoms which were most marked in rats receiving all three compounds

Toxicity of Adrenaline

From 32 mice receiving repeated injections of adrenaline, but no pyro catechol, one half were dead in 185 minutes From the same number of mice receiving pyrocatechol as well as adrenaline 50% had died in 110 minutes Owing to the great distribution of survival times the difference was not significant when tested as prescribed by WILCOXON (1945)

Discussion

Like iproniazid, pyrocatechol inhibits the depletion by raunescine of the noradrenaline in the brain of rats (PAASONEN & KARKI 1959) As already mentioned, a rise in brain 5 HT is produced by raunescine after iproniazid pretreatment (PAASONEN & KARKI 1959) An increase in brain noradrenaline content is produced by raunescine only when it is given after pretreatment with both iproniazid and pyrocatechol The probable explanation for this difference is that though iproniazid alone inhibits mono amine oxidase, the main metabolising enzymes of 5 HT – both monoamine oxidase and catechol O methyltransferase – have to be blocked to produce the same effect on noradrenaline This agrees with the recent concept of catechol amine metabolism (ARMSTRONG & McMILLAN 1957, AXELROD 1957, LABROSSE, AXELROD & KETY 1958, AXELROD & LAROCHE 1959) It remain to be seen whether the increase of noradrenaline is due to its protection from metabolism after it has been liberated by the Rauwolfia alkaloid from cellular binding sites (for discussion cf PAASONEN & KARKI 1959)

Pyrocatechol itself is unable to increase brain noradrenaline This may be due to incomplete inhibition of O methyltransferase Owing to the toxicity of pyrocatechol it is not possible to increase the dosage On the other hand, the time interval used, 2 hours may not have been long enough to produce a demonstrable effect

Among a number of compounds examined by GARATTINI GIACHETTI, PIERI & RE (1960), adrenaline and noradrenaline were the most active in exerting an antireserpine effect on eyelid ptosis in mice Iproniazid had no effect It is likely that in our experiments the eyeopening effect of the combined treatment by iproniazid and pyrocatechol was due to the

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The Degree of Polymerisation of Hyaluronic Acid Isolated from the Myometrium of the Human Cervix Uteri

By

Ole H Iversen and K Marcker

(Received January 14 1961)

A previous paper (IVERSEN 1960) recorded the isolation of acid mucopolysaccharides from the human cervix uteri, the findings suggested the presence of hyaluronic acid and chondroitin sulphate A and B in the isolated substances, as well as a variation with age and androgenic treatment in the degree of polymerisation or the relative amount of hyaluronic acid

In the work described here the degree of polymerisation of hyaluronic acid isolated from the human cervix uteri has been determined by osmometry

Material and Methods.

53 cervix uteri, removed by operation (Department of Gynaecology, Bispebjerg Hospital), were comminuted and stored in acetone for at least a month None of the patients had formerly been treated with cortisone, thyroid or corticotropin The operative indications were fibromas of the corpus uteri, adnexal complaints and – in a few instances – anomalies in bleeding There were not found any important macroscopical or histological abnormalities in either the myometrium or the endometrium of the cervix

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The cyclical phase was determined on all [illegible]

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For the purpose of *isolating the mucopolysaccharides* we mainly used the method previously described (IVERSEN 1960), but certain modifications were made and the method will therefore be briefly described To obtain sufficient amounts of material the preparations were pooled in groups of 4-5 The dried, de fatted tissue was incubated for 72 hours with barbital buffer pH 7 8, to which were added tetracemin (edet c acid, Versene ®) 3 24 g/l After precipitation with trichloroacetic acid and dialysis the filtrate was evaporated to a volume of 50 ml, and the residual protein split products were removed by repeated shaking with chloroform and isoamylalcohol (SEVAG 1934) After dialysis for 48 hours, the mucopolysaccharides were precipitated with 3-4 vol cold alcohol In order to remove the sulphated polysaccharides a solution of the substance in 0 1 M-NaCl was brought to pH 1 5 by means of HCl at 0°C with vigorous stirring Mesh amine ion-exchange resin dovex ® in the chloride form was added and the mixture was stirred for 2 hours at 0° The resin was removed by centrifugation and subsequent filtration through sintered glass funnels, until the solution was perfectly clear After removal of the resin, the solution was brought to pH 7 with NaOH and then precipitated with 2-3 volumes of alcohol (SIMMONS 1955) The precipitate was washed in alcohol and ether, dried under reduced pressure until constant weight was obtained, and weighed The isolated substance was a fine white or yellowish white powder, hygroscopic, fairly easily soluble in water

The *molecular weight* was calculated from osmotic pressure determinations obtained by the micro osmometer described by CHRISTIANSEN and JENSEN (1953) The concentration used in these experiments was 5 mg of the isolated substance in 1 ml 0 5 M potassium chloride according to our experience the Donnan effect is then negligible

The sulphur analysis was performed after the method of ZINNEKE (1951)

Nitrogen determination was done by Dumas' method

Viscometrical measurements were made in the micro viscometer described by DALGAARD-MIKKELSEN and KVORNING (1948)

Results.

The assumption that the isolated material is hyaluronic acid with minor impurities is based on five considerations 1) The material is white or yellowish-white 2) it is fairly easily soluble in water 3) the solution is viscous, the viscosity decreasing on addition of bacterial hyaluronidase and giving the characteristic degradationcurve of hyaluronate 4) a low content of sulphur, indicating minor amounts of chondroitin sulphates 5) a nitrogen percentage only a little higher than the theoretical (3 4%)

During the experiments it was found that the yield of hyaluronic acid was about 0 75% of dried defatted tissue, the largest amounts from the younger individuals and from hormone-treated patients, the smallest amounts in the individuals of 40 years or more Further, it was found that the amount of isolated hyaluronic acid corresponded to about 55% of the total mucopolysaccharides isolated, the largest amounts being found in the younger and hormone treated individuals

The results of the investigation are shown in table 1 The grouping was

Table 1

Molecular weights (M) of hyaluronate isolated from the myometrium of the human cervix uteri calculated from measurements of osmotic pressure

Age	Cyclical phase	Hormonal treatment	$M \times 10^3$	Per cent N	Per cent S
20–30	P		68	–	0.92
20–30	P		63	–	0.83
30–40	P		67	–	–
40–50	P		62	–	–
40–50	S		82	3.98	0.36
40–50	S		48	3.65	0.59
40–50	S		48	–	0.44
40–50	P	androgenic	57	–	1.02
40–50	S	androgenic	54	4.02	0.47
40–50	S	androgenic	52	–	1.89
30–40	P	estrogenic	62	–	–
40–50	P	estrogenic	48	–	–
40–50	S	estrogenic	65	4.22	0.79
30–40	P	estrogenic	66	–	0.44
>60			34	3.82	1.03
>60			41	–	–

P proliferative phase S secretory phase

according to age and cyclical phase, as well as to treatment with oestrogen or androgen It appears that no significant variations were found in the molecular weight of the substance

Discussion

The molecular weights shown in table 1 refer to apparent, not too true, molecular weights The latter, obtainable by extrapolating to zero concentration, are therefore a little higher than those given in the table The isolation procedure employed in this investigation is rather mild, and it is not likely to have been responsible for any depolymerisation worth mentioning It is therefore concluded that the degree of polymerisation of hyaluronate isolated from the myometrium of the human cervix uteri is fairly low as compared with hyaluronic acid isolated from other tissues (JENSEN 1957, BLIX & SNELLMANN 1945, ZACHARIAE 1958, SYLVÉN & MALMGREN 1952, BLUMBERG & OSTER 1954, HVIDBERG 1959)

No significant variations were found in the molecular weights The previously found variations in the viscosity depending on age and androgenic treatment (IVERSEN 1960) must therefore have been due to changes in the relative amounts of hyaluronic acid and chondroitin sulphate This also is in accordance with the work of BERTHELSEN & MARCKER (1960), who found variations in the ratio HA/C of aortic tissue with age

Summary.

Acid mucopolysaccharides were isolated from the myometrium of the human cervix uteri. The hyaluronic acid was isolated by adding ion-exchange resin. The molecular weights were determined by micro-osmometry; no significant variations in the molecular weights were found dependent on age or on androgenic or oestrogenic treatment.

Acknowledgements.

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The N and S per cent has kindly been determined by Leo pharmaceutical products, microchemical laboratory.

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From the Department of Pharmacology, Royal Veterinary College, Stockholm, Sweden

Pharmacological Actions of Aprobit® – a long acting Antihistaminic Compound without sedative Effect

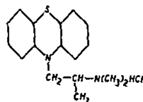
By

L. Albanus, E. Hansson, C. G Schmiterlow

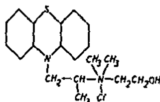
(Received December 7, 1960)

Promethazine has gained widespread clinical use, with many different indications The marked antihistaminic effect of the drug is responsible for its use in treating allergic disorders As an antiallergic drug, however, it has limitations because of its marked sedative action We therefore thought it would be of interest to try to separate sedative and antihistaminic actions

Promethazine (WHO, NFN) (N (2 dimethylamino-2 methylethyl) phenothiazine chloride) is a tertiary phenothiazine compound with hydrophilic properties The solubility in water is however easily lost under the slight alkaline conditions that occur in the tissues Promethazine enters easily into the central nervous system (SCHMITERLOW 1956), and it could be expected that the sedative effect would disappear if the hydrophilic properties were increased to a point at which the solubility in water persisted even in an alkaline medium



PROMETHAZINE



APROBIT®

On this working hypothesis a series of quaternary phenothiazine derivatives were synthetised Of those tested so far, the substance 1 (10-phenothiazinylmethyl)ethyl-2 hydroxy ethyldimethylammonium chloride

(Aprobit ®, Recip Co, Stockholm, Sweden) showed an antihistaminic activity similar to that of promethazine As, however, it had been found in preliminary experiments that aprobit lacked the sedative properties of promethazine, it was thought desirable to investigate aprobit more thorougly and to examine in more detail the possible differences between the two drugs

Methods.

Antihistaminic Action

The antihistaminic activities of aprobit and promethazine were tested on guinea p g ileum, on the histamine aerosol effect in guinea pigs and on the bronchial tone of guinea pigs

On ileum The antihistaminic action was tested on isolated guinea pig ileum in oxygenated Locke's solution at 37°C, and antihistaminic activity was calculated as described by SCHMITERLOW (1948)

On histamine aerosol The method employed was a modification of the technique of HALPERN (1942)

Guinea pigs weighing 190–210 g were used Each animal was placed in a glass container, and the aerosol was made with 0 05% histamine chloride in glycerol water (1 4) solution Aprobit or promethazine was given intramuscularly from 30 minutes to 16 hours before the animals were exposed to the aerosol If the animals could stay for 5 minutes in the aerosol without showing any signs of dyspnoea, they were con sidered to be protected

On bronchial tone Bronchial tone was recorded by the method of KONZETT & RÖSSLER (1940) The guinea pigs were anaesthetised with urethane, and the lungs were artificially ventilated by a Starling pump, which drove air into the trachea Tracings were made by using a piston recorder on smoked paper

Antiacetylcholine Action

The anti acetylcholine actions of aprobit and promethazine were tested on guinea pig ileum

Blood Pressure

Blood pressure was recorded in cats under chloralose urethane anaesthesia with a mercury manometer connected to the carotid artery Injections were made via the femoral vein

Isolated heart

The rabbit heart was excised and a cannula was introduced into the aorta above the semilunar valves Oxygenated Locke Ringer solution was perfused through the coronary vessels at a constant temperature (38°) The contractions were recorded by a lever attached to the apex of the ventricles The coronary flow was measured by collecting the perfusate in a Gaddum s outflow recorder and registering the volume over constant intervals of time

Effects on Blood Vessels

The effect of aprobit on blood vessels was studied on isolated rabbit ear or hind leg Ringer solution was perfused into the central artery of the ear or the femoral artery The amount of perfusate collected from the veins was measured at constant intervals of time

Action on Gastric Motility

electrode was placed on the peripheral part of the left cut vagus in the neck Blood pressure was recorded from one of the carotid arteries

Ganglionic blocking Action

The contractions of the nictitating membrane of cats under chloralose-urethane anaesthesia were recorded mechanically on a kymograph The preganglionic fibres of the cervical symphathetic trunk were stimulated electrically by means of two silver electrodes in a polymethacrylate rod The stimuli were applied for 20 secs at intervals of 3 minutes The electrical stimulus had the characteristics· pulse frequency 10 per sec, pulse duration 15 msecs, strength 1 5–2 0 V

Respiration

The effect of aprobit on respiration was studied in chloralose-urethane anaesthetised, and on decerebrate cats by body plethysmography The skin was cut around the neck just behind the ears and mobilised from the neck and a tracheotube was inserted The cat was then put into a metal case The head of the cat was pulled out of the case through a round hole surrounded by a collar The mobilised skin was turned over the collar and fixed tightly around it

The case was connected by a rubber tube to a float recorder writing on a smoked drum, calibrated so that the respiration volume could be calculated

The advantage of this method is that the neck of the cat is free and other records, such as blood pressure from the carotid artery and stimulation of the nictitating membrane, can easily be made Injections were made through the jugular vein

Toxicity Experiments

Acute toxicity was determined in mice by subcutaneous and oral routes Deaths were counted after 48 hours and the LD50 was calculated by the procedure of BLISS (1952)

Subchronic toxicity was studied in rats A group of 7 male rats received on six days a week for 3 weeks an oral dose of 50 mg/kg aprobit given in approximately 0 5 ml water An equal number of animals was given the same amount of water The rats were weighed twice a week At the end of the experiment the rats were killed for macroscopic and microscopic pathological examination

Chronic toxicity was studied in rabbits The rabbits were given oral doses of 50 mg kg aprobit in gelatine capsules 6 days a week for eight weeks The controls received the corresponding amounts of empty gelatine capsules The rabbits were weighed twice a week At the end of the experiments the rabbits were killed for macroscopic and microscopic pathological examination

Central depressive Effects

Effect on barbiturate narcosis The technique was a modification of that of COURVOISER *et al* (1953) A hypnotic intraperitoneal dose of 35 mg/kg thiopental sodium (thiomebumalnatrium NFN) or 75 mg/kg hexobarbital sodium (enhexymalnatrium NFN) was administered to rats of five groups The sleeping time of the animals in control groups receiving no premedication was compared with that of animals given intramuscular aprobit or promethazine 30 minutes before the barbiturates

Conditioned avoidance escape in rats The conditioned avoidance escape response was studied in rats The experimental technique was basically that of COOK & WEIDLEY (1957) The animals were first conditioned to avoid an electric shock by placing them in a box whose floor is a grid composed of stainless steel rods Shocks of 40 mA and 60 V were delivered to the grid from a stimulator The rats escape the shock by climbing a pole in the centre of the box A buzzer was used as conditioning stimulus The rats were trained for about 3 weeks before the actual experiment

Climbing test in mice The effect of aprobit and promethazine on the activity of mice was tested by the mouse climbing test method of KNEIP (1960) Mice weighing 19–21 g were kept in a small cage containing a small ladder The number of mice climbing the ladder was recorded 1, 2, 4, and 8 hours after administration of the drug

Spontaneous aggressiveness in Siamese fighting fish (Betta splendens) The effect of the drugs on spontaneous aggressiveness was tested on sexually mature male fish by the method described by WALASZEK & ABOOD (1956) The fish were placed in an experimental aquarium divided into two parts by a transparent watertight window and a removable opaque slide The fish were observed for five minutes, and the number of attacks by each fish was noted The opaque slide was then put in place, and to one of the chambers the drug to be tested was added in aqueous solution and allowed to act for 30 minutes After that the slide was taken away, and the fish were observed for another five minute period

Results.

Antihistaminic Action.

The relative antihistaminic actions of promethazine and aprobit on the guinea pig ileum were compared The results are shown in fig 1 The antihistaminic action of aprobit was similar to that of promethazine

The protective action of aprobit against histamine aerosol in guinea pigs is shown in table 1 For comparison we also measured the antihistaminic activity of promethazine Both aprobit and promethazine protected guinea pigs against the histamine aerosol at a dose of 1 mg/kg For both drugs the dose that abolished the histamine shock was the same as was their duration of action The effect of aprobit on the bronchioles studied by the method of Konzett & Rossler is shown in fig 2 Aprobit abolished the constrictive effect of histamine on the bronchioles

Antiacetylcholine Action

The anti-acetylcholine activities of the two drugs were tested on guinea pig ileum Fig 3 illustrates the dose-response curve for aprobit and promethazine in reducing the response of the gut to a standard dose of acetylcholine The anti-acetylcholine effect of aprobit was found to be slightly stronger than that of promethazine

Action on the cardiovascular System

Doses of more than 1 mg/kg intravenously of aprobit produced a fall in blood pressure, which developed almost immediately and was maximal

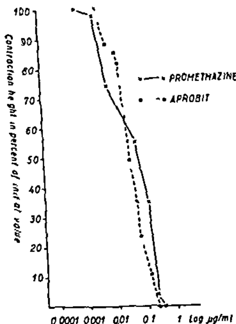


Fig 1 The effect of promethazine and aprobit on histamine induced contractions of guinea pig ileum

1 minute after the injection This depressor action of aprobit was, however, of short duration, the blood pressure returning to its original level 2–3 minutes after the injection (fig 4) No hypotensive effect was observed in animals when 20 mg/kg aprobit was given intramuscularly From fig 4

Table 1

The action of aprobit ® and promethazine on bronchiospasms in guinea pigs induced by histamine aerosol

Drug	Dose	Number of animals protected out of total number of treated animals at various intervals after administration of the drug					
		30 min	1 h	2 h	4 h	8 h	16 h
Aprobit	1 mg/kg	8/8	8/8	4/8	2/8	*	*
Aprobit	2 mg/kg	10/10	10/10	10/10	8/10	x	x
Aprobit	4 mg/kg	8/8	8/8	8/8	7/8	5/8	4/8
Aprobit	8 mg/kg	8/8	8/8	8/8	8/8	5/8	5/8
Promethazine	1 mg/kg	8/8	8/8	4/8	3/8	*	*
Promethazine	2 mg/kg	8/8	8/8	8/8	7/8	*	*
Promethazine	4 mg/kg	8/8	8/8	8/8	6/8	3/8	2/8
Promethazine	8 mg/kg	8/8	8/8	8/8	8/8	7/8	3/8

*) Not investigated



Fig. 2. The effect of aprobit on bronchiolar tone of guinea pig recorded by method of Konzett & Rössler (Hi = histamine)

it is also evident that aprobit exerted a marked adrenolytic action. The pressor effect of noradrenaline was not affected. Promethazine in identical doses did not cause this adrenaline reversal.

Aprobit abolished the hypotensive effect of histamine. A dose of 1 mg/kg

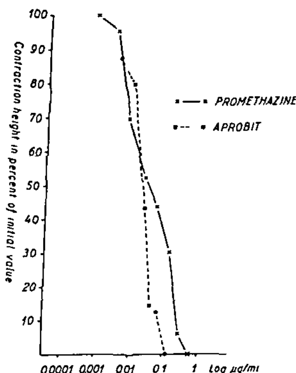


Fig 3 The effect of promethazine and aprobit on acetylcholine-induced contractions of guinea pig ileum

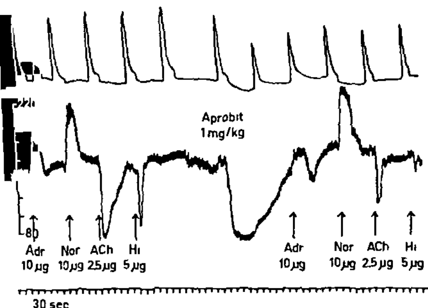


Fig 4 Cat 2.5 kg Chloralose-urethane Upper curve contractions of the nictitating membrane due to preganglionic stimulation Lower curve carotid blood pressure (Adr = adrenaline Nor noradrenaline Hi histamine ACh = acetylcholine)

body weight of aprobit is enough to reduce the blood pressure lowering effect of 5 µg histamine (fig 4)

It was also found that 1 mg/kg aprobit reduced the depressor effect of a given dose of acetylcholine by approximately 50% This anti acetylcholine effect is slightly greater than that of the identical dose of promethazine

Aprobit was administered into the cannula of isolated heart preparation at various concentrations The drug did not produce any change in coronary flow or any observed effect on the amplitude of contractions or heart rate

Because of the depressor action seen after intravenous injection of aprobit the effect of the compound on the vessels of the perfused isolated hind limb or ear of the rabbit was determined Doses of up to 10 mg per ml of aprobit were injected into the perfusion cannula Neither vasodilator nor vasoconstrictor effects were observed

Action on Gastric Motility

On stimulation of the peripheral end of the vagus a fall in the blood pressure and strong contractions of the stomach were observed After

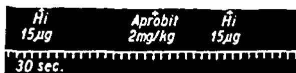


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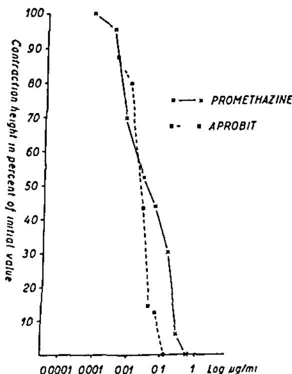


Fig 3. The effect of promethazine and aprobit on acetylcholine induced contractions of guinea pig ileum

partly responsible for this increase in respiration, but not entirely, since promethazine at the same dose caused an almost identical drop in blood pressure without any increased respiration, indeed, promethazine usually caused a temporary apnoea Further the blood pressure fall caused by aprobit was, sometimes negligible, though the effect on the respiration persisted

Toxicity Experiments

Acute toxicity The LD50 in mice was found to be 266 mg per kg body weight subcutaneously and 575 mg per kg body weight orally

Subchronic toxicity Chronic feeding for three weeks failed to produce any significant departure from the control growth curves of rats Gross examination as well as histological sectioning of liver and kidney at the end of three weeks feeding did not reveal any changes

Chronic toxicity After chronic feeding of rabbits for eight weeks, no significant difference was observed in the growth of any of the test animals and the controls Histological examination was made of liver, kidney, spleen and bone marrow No histopathologic lesions attributable to Aprobit were noted

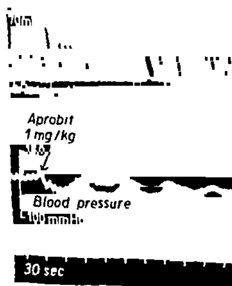


Fig 6 Cat. Chloralose urethane Upper curve respiration recorded with a body plethysmograph Lower curve carotid blood pressure

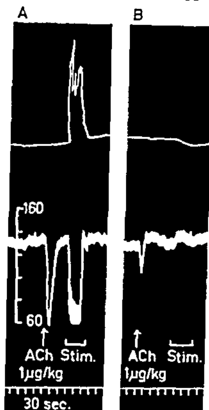


Fig 5 Cat Chloralose urethane Upper curve gastric motility Lower curve arterial blood pressure Stimulation of the left vagus nerve
A before and B after 1 mg/kg aprobit

injection of aprobit, stimulation of the vagus did not cause any fall in blood pressure or contractions of the stomach (fig 5)

Ganglionic blocking Action

An intravenous injection of 1 mg/kg aprobit caused a temporary reduction in response of the nictitating membrane to preganglionic stimuli This partial block was rapid in onset The duration of aprobit action was relatively short, the blocking effect disappeared only a few minutes after the injection (fig 4) With 2 mg/kg a total though still temporary block was obtained

Effect on Respiration

In cats under chloralose urethane anaesthesia, as well as in decerebrate cats, aprobit at a dose of 2 mg/kg caused a marked increase in respiration (fig 6) Both respiration frequency and amplitude were increased Under the influence of aprobit the response to a high percentage of CO_2 (approx 8%) in the inhaled air was markedly exaggerated The fall in blood pressure caused by injection of the drug could be

Table 2

Effect of aprobit and promethazine on spontaneous activity in mice

Drug	Dose *	Number of animals tested	Number of animals climbing the ladder out of total number of treated animals at various intervals after administration of the drug			
			1 h	2 h	4 h	8 h
Controls		20	20/20	19/20	19/20	20/20
Aprobit	10 mg/kg	20	20/20	18/20	19/20	19/20
Aprobit	25 mg/kg	20	18/20	20/20	19/20	20/20
Promethazine	10 mg/kg	20	12/20	16/20	16/20	18/20
Promethazine	25 mg/kg	50	5/50	8/50	32/50	38/50

Promethazine shows closely similar activity but its effectiveness was found to be lower than that of chlorpromazine higher doses being necessary to give the same response A dose of approximately 10 mg/kg promethazine had about the same effect as 2 mg chlorpromazine

Aprobit on the other hand failed to produce any block of the conditioned reflex

Climbing test The percentage of mice that failed to climb the ladder at different times after the administration of the drugs is seen in table 2 The administration of 10 mg/kg promethazine had a slight effect but at a dose level of 25 mg/kg promethazine the effect was marked only 10% climbed the ladder 1 hour after the injection There were no obvious effects on the climbing of the mice after administering the same doses of aprobit

Spontaneous aggressiveness in Siamese fighting fish Promethazine had a marked effect on the spontaneous aggressiveness seen in the Siamese fighting fish A dose of 10 mg per litre abolished the fighting response Contrary to the effect observed after barbiturates the fish still retained its flight reflex when treated with promethazine After doses up to 20 mg per litre of aprobit the spontaneous aggressiveness was quite unaltered

Discussion

The quaternary phenothiazine compound aprobit ® first described by CARLSSON HANSSON NILZEN & SCHMITERLOW (1960) has been subjected to a more detailed pharmacological study This revealed some similarities and some differences between aprobit and the well known tertiary phenothiazine compound promethazine

The antihistaminic properties of the two drugs were found to be almost identical both inhibiting the action of histamine on intestinal and bronchiolar smooth muscles and both also preventing the effect of histamine in reducing blood pressure Aprobit was also found to be a potent anti acetylcholine drug showing blocking effects not only on effector structures with postganglionic cholinergic innervation but also on synaptic transmission This action may be due to the fact that a choline like structure exists in the side chain of aprobit and may enable this drug to compete effectively with acetylcholine for receptor sites

Aprobit abolished the effect of vagal stimulation on the motility of the stomach This is in accordance with the findings of GERNANDT & SCHMITERLOW (1953) who showed that promethazine exerted this effect and suggested that this probably accounts at least partly for its well known anti-emetic effect in the treatment or prevention of motion sickness The blocking effect of aprobit was even more striking which strongly suggests that this drug could also be used in motion sickness

Central depressive Effects.

Gross behavior The effect on the gross behavior of rats was observed more or less continuously for a period of 8 hours after intramuscular administration of aprobit or promethazine Promethazine had a strong sedative effect on the rats, and at dose levels up to 50 mg/kg body weight both sedation and ataxia were grossly observable effects There were no obvious effects after administration of up to 50 mg/kg body weight of aprobit

Effect on barbiturate narcosis The effects of aprobit and promethazine on sleep induced by barbiturate were studied in rats Aprobit had no effect on the sleeping time of rats injected with hexobarbital or thiopentone, whereas the same doses of promethazine increased the sleeping time

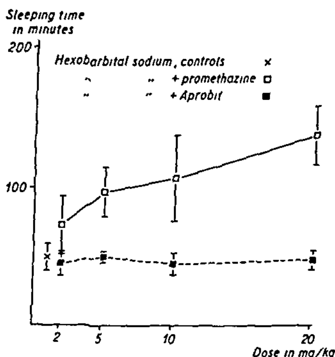


Fig 7 The effect of promethazine and aprobit on sleeping time produced by hexobarbital (enhexymal) in rats

In fig 7 are shown the results of increasing doses of promethazine and aprobit on sleep caused by barbiturate The sleeping time increased almost linearly with dose in animals pretreated with promethazine

Conditioned reflexes Chlorpromazine was given as a standard drug to groups of previously conditioned rats, in order to study the disappearance of the conditioned avoidance escape response After a dose of 2 mg/kg chlorpromazine the conditioned reflex was blocked in 50% of the rats

being a tertiary and aprobit a quaternary compound This difference may account for the differences in some of their actions, particularly in their effects on the central nervous system Promethazine, being a tertiary compound, is easily soluble in lipids after alkalisation, whereas the quaternary compound aprobit is not lipid soluble As has been pointed out by Brodie & Hogben (1957), there exists a close correlation between the distribution of drugs and their lipid solubility in the un ionized form The blood brain barrier offers an exceptionally good illustration of the close connection between lipid solubility and permeability through biological membranes

Summary.

The pharmacological properties of the quaternary compound 1-(10-phenothiazinylmethyl)ethyl-2 hydroxyethyldimethylammonium chloride (aprobit ®) are described The investigation includes a comparison between this drug and the tertiary phenothiazine derivative, promethazine

Aprobit is a potent antihistaminic compound, action and duration being similar to those of promethazine

Aprobit has a marked adrenolytic effect not produced by promethazine in corresponding doses

Aprobit causes a stimulation of the respiration not caused by promethazine

Aprobit, like promethazine, abolishes the gastric contractions caused by stimulation of the vagus

The toxicity of aprobit is low

Aprobit does not show a sedative action on the central nervous system as does promethazine

The reasons for the observed similarities and differences in effect between these two phenothiazine compounds are discussed

In conclusion it can be said that aprobit is an effective, long-lasting, non toxic, antihistaminic compound lacking sedative effects

Acknowledgement

The skilled technical assistance of Mrs G Persson and Mrs E Engqvist is gratefully acknowledged

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In the further study of the pharmacodynamic action of aprobit on the autonomic nervous system, it was found that it had a marked adrenolytic action and also a ganglion-blocking effect, two qualities absent from promethazine The adrenolytic action is difficult to explain, but shows the usual pattern, including a reversal of the effect of adrenaline on the systemic blood-pressure but little effect on the rise in blood pressure caused by noradrenaline The ganglionic blocking effect may be due to the above mentioned choline-like structure in the aprobit molecule

Aprobit blocked the effect of vagus stimulation on both circulation and the stomach It was found, however, that the effect of injected acethyl choline was more difficult to abolish It is generally agreed that it is easier to block the effect of exogenous acetylcholine than that of vagus stimulation This was obviously not so with aprobit, the reason perhaps being that this drug exerted a blocking effect at both the postganglionic and the preganglionic cholinergic structures

The respiratory stimulation caused by aprobit could be interpreted in several ways The effect must obviously be a peripheral one, as HANSSON & SCHMITERLÖW (1960) have shown that aprobit does not penetrate into the brain The fall in blood-pressure, causing a stimulation of the receptors in the carotid region, may well account for at least a part of the increase in respiration Further investigations, including a record of the action potentials in the sinus nerve, are considered to be necessary before any definite conclusions can be drawn about this mechanism In this connection it should be pointed out that promethazine, although it caused an equidepressor effect on blood pressure, did not cause an increase in respiratory frequency or amplitude – on the contrary there was a transient apnoea

The main difference between the actions of promethazine and aprobit was established when that on the central nervous system was studied The depression caused by promethazine was entirely absent with aprobit The gross behaviour of the animals was not influenced even by large doses of aprobit The conditioned avoidance-escape reaction was not affected, spontaneous aggressiveness in the male *Betta splendens* persisted and barbiturate-induced sleep in rats was not prolonged when aprobit had been given This difference between promethazine and aprobit is explained by the fact that promethazine easily enters into the brain, whereas aprobit cannot penetrate the blood-brain barrier (SCHMITERLÖW 1956, HANSSON & SCHMITERLÖW 1960)

The structure of the two compounds, promethazine and aprobit, is to a certain extent rather similar, both being derivatives of phenothiazine This may account for the similarity in some of their actions On the other hand there is also a striking difference in their structure, promethazine

being a tertiary and aprobit a quaternary compound This difference may account for the differences in some of their actions, particularly in their effects on the central nervous system Promethazine, being a tertiary compound, is easily soluble in lipids after alkalisation, whereas the quaternary compound aprobit is not lipid soluble As has been pointed out by BRODIE & HOGBEN (1957), there exists a close correlation between the distribution of drugs and their lipid solubility in the un ionized form The blood brain barrier offers an exceptionally good illustration of the close connection between lipid solubility and permeability through biological membranes

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Acta pharmacol et toxicol 1961, 18, 119–128

From the Pharmacological Laboratory of DUMEX, Ltd, Copenhagen
(E Jacobsen, M D)

The Determination of Noscapine (Narcotine) in Plasma and Urine.

By

Sven Vedsø

(Received January 30 1961)

Noscapine (formerly known under the falsely descriptive name of narcotine) is an alkaloid present in opium in amounts only exceeded by those of morphine It has been known for almost 150 years and was first isolated in 1817 by Robiquet However few pharmacodynamic effects of this substance were established, and it was considered as almost inert until it was recently discovered that noscapine possesses pronounced antitussive properties (KONZETT & ROTHLIN 1954, BICKERMAN & BARAC 1954), which revived interest in noscapine and its pharmacology This paper describes a sensitive and specific method for determining the alkaloid in plasma and urine

The many methods given in the literature for quantitative determination of opium alkaloids are based on various principles Unfortunately the sensitivity of these methods is too low to permit the determination of opium alkaloids including noscapine, in plasma or urine after oral or parenteral administration, because the doses that can be used generally result at concentrations below 1 μg/ml

COOPER & HATCHER (1934) used Valser's reagent (a solution of potassium mercuric iodide) for determining small amounts of noscapine The reagent reacts with noscapine and produces a fluorescence by means of which amounts down to 1 μg can be measured The two authors investigated the distribution and excretion of noscapine in cats after intravenous administration, but the lack of sensitivity of the analytical method led to only approximate results Nevertheless [illegible] been described by WHITMORE & V [illegible] VIL *et al* (1945) and VIDIC (1952) [illegible]

Courvoisier, S, J Fournel, R Ducrot, M Kolsky & P Koetschet Propriétés Pharmacodynamiques du Chlorhydrate de Chloro 3(Diméthylamino 3 Propyl) 10 Phénothiazine (4 560 R P) *Arch int pharmacodyn* 1953 *92, 305–331*

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which noscapine can be determined in plasma and urine at concentrations down to 50 ng/ml (1000 ng = 1 μg)

The pH of the plasma or urine is adjusted to 10 by adding alkali, and the noscapine base is taken up in an ether phase, which is then extracted with dilute hydrochloric acid The acid extract is adjusted to pH 9 2, whereby the greatest increase in fluorescence is obtained (fig 1) The fluorescence is determined before and after heating for 30 minutes at 120° in an autoclave The corresponding increase of fluorescence of a series of standard solutions of noscapine treated in the same way is also measured, and the content of noscapine in the test solution is thus calculated

Methods

Apparatus

Centrifuge tubes with conical ends 10 ml, furnished with Quickfit No 14 glass stoppers greased with Dow Corning silicone grease

Test tubes diameter 16 mm

Aluminium foil

2 Carlsberg constriction pipettes 2 ml

2 Carlsberg constriction pipettes 1 ml

Shaking apparatus, shaking horizontally with excursions of 2 cm and 300 shakings per minute

Autoclave (120°)

Fluorimeter – able to give full scale deflection for a solution of 10 [illegible]

[illegible]

Reagents

All reagents are prepared from freshly glass distilled water

1) S[illegible]

sto[illegible]

wa[illegible]

2) 1 N NaOH

3) 0 1 N HCl prepared daily from 1 N HCl

4) Buffer reagent I pH 10 0 Equivalent amounts of $NaHCO_3$ (21 0 g) and Na_2CO_3 (26 5 g) are mixed washed 3 times with re distilled ether on a filter funnel and d[illegible] heating at 30°C [illegible]

[illegible]

7) Na_2SO_4 repeatedly washed with ether and dried at 110°

8) Two solutions of [illegible] ulphate in 0 1 N H_2SO_4 containing 14 μg and 70 μg

[illegible]

cipitating the alkaloid from an acid solution with alkaloidprecipitating reagents such as silicotungstic acid or bromine All these methods are unspecific and are relatively insensitive

More recently, paper chromatographic methods have been developed (CURRY & POWELL 1954, MANNERING *et al* 1954, BETTSCHART & FLUCK 1956) By means of them the opium alkaloids can be separated and demonstrated in amounts down to a few µg These methods are, however, also not sufficiently sensitive to permit quantitative determinations in plasma and urine after administering opium alkaloids in vivo

MILTHERS (1958) describes a polarographic method for morphine in blood and plasma by which morphine can be determined with great accuracy at concentrations as low as 1 µg/ml

BETTSCHART & FLUCK (1956) observed that spots of morphine nos capine and narceine in the chromatograms are fluorescent and that this fluorescence is increased considerably after heating at 120° (for 20 minu tes) The method for determining noscapine described here is based on this observation The greenish-blue fluorescence developed by heating noscapine was found to be consistent and of the same intensity as that of quinine, this has permitted development of an analytical method by

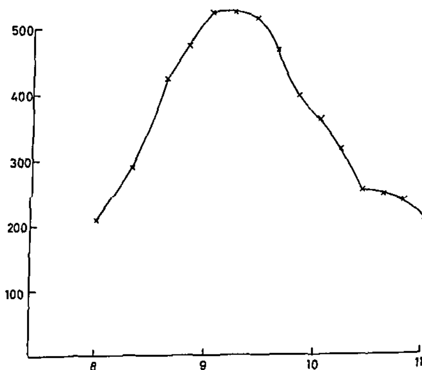


Fig 1 *Influence of pH upon increase of fluorescence*
Increase of fluorescence of aqueous solutions of 2 µg noscapine chloride/9 ml after heating to 120° for 30 min at various pH values
Abscissa pH Ordinate Increase of fluorescence

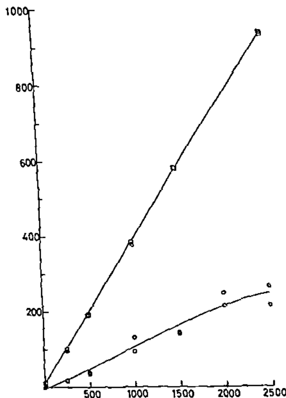


Fig 2 *Standard curve for noscapine chloride (and morphine)*

□——□ Increase of fluorescence for aqueous solutions of noscapine chloride at pH 9 2 after heating

o——o Increase of fluorescence for aqueous solutions of morphine at pH 9 2 after heating as described for noscapine chloride

Abscissa ng noscapine chloride (morphine) in sample (6 9 ml)
Ordinate Increase of fluorescence

From the increase in fluorescence of a given test solution the content of noscapine chloride can be read from the standard curve The content of noscapine chloride per ml plasma is then calculated from the formula.
Noscapine chloride in plasma (urine) =

$$\frac{\text{content read from the standard curve} \times 6.5}{5.00} \text{ ng/ml}$$

It is important to use scrupulously clean utensils and to prepare all reagents from freshly glass-distilled water, avoiding contact with rubber, which would increase the initial fluorescence considerably

The aperture for the exciting beam of the fluorimeter is kept constant during each whole series of measurements Before the measurements of

per liter The solutions can be stored at room temperature in Pyrex glass bottles for several months However, it is advisable to compare each freshly made solution with that previously used before it is finally used up

Procedure

Measure 1 00 ml urine or plasma containing not more than 2500 ng noscapine into a centrifuge tube Add 6 50 ml ether and 0 10 g buffer reagent I, fix the stopper and shake immediately in order to avoid adherence of the carbonate to the bottom of the glass Place the glass horizontally in the mechanical shaking apparatus and shake for 30 minutes Separate the two phases (centrifugation is generally not necessary) and remove the water phase from the ether phase with a 1 ml constriction pipette Dry the ether phase by shaking with 0 25 g Na_2SO_4 Transfer 5 00 ml of the dried ether phase into another centrifuge tube containing 2 00 ml of 0 1 N-HCl Place the tubes in a water bath at 18° for 5 minutes, shake by hand for 2 min , allow to separate and transfer the hydrochloric acid layer into a test tube with a 2 ml constriction pipette Care must be taken that no ether is transferred with the hydrochloric acid Repeat the extraction with 2 00 ml 0 1 N-HCl, and wash with 1 00 ml distilled water for 1 minute To the combined extracts in the test tube add 0 40 ml 1 N-NaOH and 1 50 ml buffer reagent II and mix

The fluorescence of the water phase is measured at 22° on the fluorimeter (A PHOTOVOLT Multiplier Fluorescence Meter model 540 was used) A filter isolating the 365 mμ mercury line is used for the exciting beam and one transmitting between 480 and 580 mμ as a secondary filter Two different apertures for the exciting beam can be used, depending on the strength of the fluorescence to be measured The aperture is adjusted to give full-scale deflection for two different solutions of quinine sulphate in 0 1 N-H_2SO_4, containing 14 μg/l and 70 μg/l

The test tubes are covered with aluminium foil, placed in the autoclave and heated at 120° for 30 minutes After cooling to 22° the fluorescence is measured again and the increase in fluorescence calculated

From 3–5 standard solutions containing 0–2500 ng noscapine chloride are made up as described below Of the buffer reagent II 1 50 ml are placed in a test tube 0 20 to 5 00 ml of the noscapine standard solution diluted in two steps 2000 times (= 500 ng/ml) are added, and the contents of each tube are made up with distilled water to 6 9 ml The fluorescence is measured before and after heating as described above The calculated increases of fluorescence are plotted with the content of noscapine chloride as the abscissa and the increase in fluorescence as the ordinate Within the limits of concentration prescribed (50–2500 ng) a straight line should be found (fig 2)

readings being generally from 15–25 scale units The increase after heating is also higher than those found with aqueous blank solutions, generally from 0–20 units Table 1 shows 23 double determinations of plasma from 6 different experimental subjects who during the last week had not taken any opium alkaloids A variance analysis shows that the variation of the blank values from time to time in the same person is higher than that to be expected from the analytical error ($P = 99\%$, $97\,5\% < P < 99\%$, $90\% < P < 95\%$, $90\% < P < 95\%$, $70\% < P < 90\%$, $50\% < P < 70\%$, but the variation in the blank values from individual to individual does not exceed that found from time to time within the same individual ($P < 50\%$)

Urine The analyses of urine are much less exact, because the fluorescence of the solution even before heating is high and inconsistent from sample to sample, generally more than 100 units on the scale The construction of the fluorimeter is such that the scale deflections must be multiplied by 10 when excursions from 100–1000 scale units are read As the analysis is based on a measurement of a difference in fluorescence, such high initial readings will cause considerable errors, especially when small differences, e g as seen in the blank tests, have to be measured The increase in fluorescence after heating is about 0–40 *scale units, corresponding to 0–100* ng noscapine chloride/ml urine

Table 2

Readings of fluorimeter, after heating solutions to 100°

Noscapine chloride in 15 ml	0 Min	1 hour	3 hours	5 hours	6 hours	7 hours
5 µg	13 5	77	255	550	750	800
10 µg	15	135	655	910	1250	1300
100 µg	15	1050	5250	8900	–	–

Readings of fluorimeter after heating solutions in an *autoclave to 120°*

Noscapine chloride in 15 ml	*0 Min*	10 Min	20 Min	30 Min.	60 Min
0	9 5	16	–	18	22 5
1 µg	10	110	150	160	–
2 µg	10 5	220	275	300	–
5 µg	–	–	–	750	725
10 µg		–	–	1300	1250

each series the fluorimeter is adjusted so that full-scale deflection is obtained with one of the two solutions of quinine sulphate. During the measurements this full-scale deflection must be controlled, as the light from the ultraviolet lamp is not entirely constant over long periods. At the highest sensitivity the fluorescence of the noscapine standard solutions before heating is about 10 scale-units and, as already mentioned, increases after heating in proportion to the concentration of noscapine

Blank Values

Water: When solutions without noscapine are heated as described, the fluorescence is increased 0–5 scale-units corresponding to 0–10 ng noscapine chloride/ml.

Plasma: Fluorescence of test solutions extracted from plasma containing no noscapine is somewhat higher than that found with pure water, the

Table 1.

23 blank values for plasma from six different experimental subjects

Experimental subject	Date 1960	Increase of fluorescence calculated as ng noscapine chloride ml/plasma		Average of the determinations
A	12 7	0	0	23 1
	21 7	13	9	
	18 8	19	21	
	8 9	58	47	
	26 9	45	19	
B	18 7	20	7	19 9
	26 7	13	23	
	23 8	18	11	
	21 9	65	26	
	3 11	10	6	
C	22 8	8	8	20 4
	31 8	35	28	
	6 9	26	26	
	8 9	19	13	
D	6 10	42	67	33 0
	12 10	15	21	
	20 10	26	27	
E	7 10	68	47	35 5
	15 10	0	54	
	25 10	21	23	
F	10 10	31	25	19 2
	18 10	16	25	
	28 10	5	13	

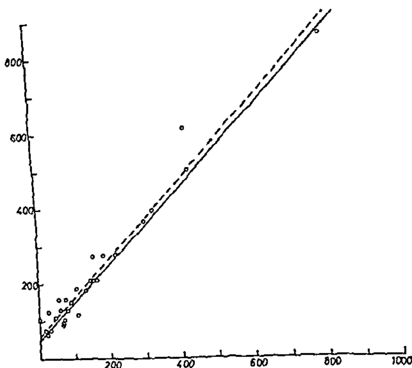


Fig 3 *Recovery of noscapine by extraction from plasma*

Abs

0

The unbroken line shows the theoretical regression line, the best fitting regression line is broken

the results of the analyses of the samples without addition of noscapine are plotted against those found in the corresponding samples with added noscapine, the former is shown as abscissa and the latter as the ordinate. A regression analysis showed that the best fitting line has a slope of 1.02 ± 0 04, crossing the ordinate at the point 62 ± 6 6 (f = 26). This line does not deviate significantly from the theoretically expected line with a slope of 1 00, crossing the ordinate at 50 ng The point of crossing the ordinate is slightly higher than expected (50 0), but the difference is within the limit of ± twice the calculated error.

The Specificity of the Method

To aqueous solutions of salts of morphine, codeine, narceine and papaverine at the same concentration as used for noscapine were added

Comments.

Some factors having a possible effect on the result of the analysis have been examined

Dependence on pH

The influence of pH on the increase in fluorescence after heating is seen from fig 1, showing that the biggest increase was at pH 9 2 Mixtures of 0 2 N-NaOH, 0 2 N-HCl and 0 2 N-$Na_2B_4O_7$ were used as buffers

Dependence on Heating Time and Heating Temperature

After adding buffer solution a series of test solutions with various contents of noscapine chloride were heated for various times to 100° on a boiling water bath or in the autoclave at 120° From table 2 it can be seen that the same increase in fluorescence is obtained after heating to 100° for 6–7 hours or to 120° for 20–30 minutes An increase in heating time does not affect the fluorescence

Reproducibility of the Analytical Results

This was examined on 26 pairs of determinations on plasma with a content of noscapine chloride up to 1360 ng/ml Each pair was analysed on the same day in the same series of analyses The variations from the mean were determined by the formula

$$s^2 = \frac{(x - \bar{x})^2}{f}$$

The series was divided in two equal groups, one with the determinations from 50–400 ng/ml, the other with 400–1360 ng/ml In the first group (50–400 ng/ml) the standard error of a single determination was found to be ±5 13% (n = 13), for the second group (over 400 ng/ml) ±5 55% (n = 13) The calculated combined error for both groups of analyses (50–1360 ng/ml) was found to be ±5 4% (n = 26) The relative error for determinations below 50 ng/ml was higher, because the method is based on a difference between two readings and the error of the readings on the fluorimeter has a greater effect when the differences are small

The Recovery of Noscapine by Extraction

Twenty eight random samples of plasma from individuals to whom noscapine had been administered were each divided into two portions, one of these portions was analysed directly, the other after addition of 50 ng noscapine chloride/ml dissolved in 0 1 ml distilled water In fig 3

Acta pharmacol et toxicol 1961, 18, 129–132

From the Cardiological Laboratory, Gentofte Amtssygehus, Copenhagen

Intestinal Absorption of Theophylline from an Aqueous Alcoholic Solution.

By

Mogens Jorgensen and Poul Moller

(Received February 20 1961)

That theophylline and its derivates are often effective in asthmatic attacks is doubted by few Their somewhat dramatic effect, most often experienced after intravenous administration, can hardly be surpassed on administration by any other route The absence of a physician or other person able to carry out the necessary venipuncture, however, often delays the onset of treatment Intramuscular injection of aminophylline has also been used because of its rapid effect, but this requires the assistance of at least an experienced nurse and is frequently accompanied by somewhat severe local pain

SCHLUGER MCGINN & HENNESSY (1957) have introduced an aqueous alcoholic solution of theophylline, which can be administered *orally* without unpleasant gastro intestinal side effects, and *found that this* solution gave higher theophylline blood levels than tablets *containing* equivalent amount of aminophylline and choline theophyllinate The preparation proposed by these authors contains 400 mg theophylline dissolved in 75 ml of 20% alcohol in water

SPIELMAN (1957) and SCHLUGER, MCGINN & BURBANK (1957) have reported good clinical *results with this* preparation on treating acute asthmatic attack The alcohol is supposed to enhance the absorption of theophylline from the gastro-intestinal tract

It would be an obvious practical advantage if this new method of administering theophylline *could replace* intramuscular injections The reported clinical results are promising However, we thought it of interest to compare blood *levels of* theophylline after each method of administration, if possible to establish whether the preference for the oral route is justified We *also tried* to discover whether addition of alcohol has, as mentioned above, the effect of accelerating theophylline absorption

buffer solution (pH = 9.2) treated as described and compared with standard solutions of noscapine. No initial fluorescence and no increase in fluorescence after heating were seen with codeine, narceine or papaverine. Morphine, however, gave an increase of fluorescence corresponding to nearly half that found with noscapine, but, unlike with noscapine, no proportionality was found between concentration of morphine and increase in fluorescence. However, the method could be modified so as to develop a morphine determination with a sensitivity of up to 0 1 µg/ml (fig. 2).

Summary.

A fluorimetric method for quantitative determination of noscapine in plasma or urine has been developed; the method is based upon the great increase in fluorescence occurring on heating noscapine at pH 9.2. The sensitivity of the method allows quantities of 50 ng/ml to be determined (s = ±5.4%).

The influence of various factors on the validity of the results has been examined.

Acknowledgment.

I am grateful to Miss Hanne Tvilling for valuable technical assistance.

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intramuscularly Planned experiments with an aqueous solution of theophylline on the last three persons (K, F and N) were given up because of the unpleasant side-effects experienced in the two first experiments (See below)

Venous blood samples were taken 10, 30, 60 and 120 minutes after administering the drug *Theophylline analysis* were made of whole blood immediately after the experiments Plasma was stored at $-20°C$ for alcohol determination on the following day

[illegible]

tion is determined by spectrophotometry of the sodium hydroxide fraction at 277 nm Recoveries were 91% from blood samples of known theophylline concentration prepared by adding small amounts of theophylline in aqueous alcohol or freshly made solutions of theophylline in water

Alcohol determinations were conducted with alcoholdehydrogenase

Results.

In the three experiments illustrated by fig 1 (B, F and N) the theophylline concentration was higher after oral than after intramuscular administration The higher maximum after oral intake was reached after a steeper rise in concentration than is shown by the values after intramuscular injection In the fourth experiment (K) there was no difference between the two routes of administration

Fig 2 shows a comparison between theophylline blood levels after oral administration of theophylline solution with or without alcohol, together with the associated alcohol-concentration curve The absorption

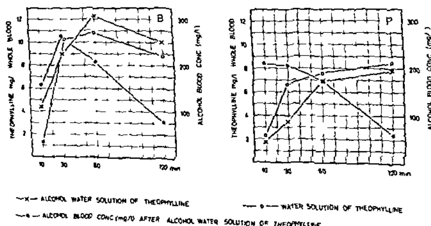


Fig 2 Blood theophylline levels and alcohol blood concentrations following oral intake of 400 mg theophylline in alcohol water solution and blood theophylline levels following 400 mg of theophylline in water solution

Methods.

The absorption experiments were performed on 5 human volunteers, 20–30 years of age, who had not taken barbiturates, analgaesics or the like for at least 24 hours and who had not had coffee or tea for at least 12 hours

The volunteers arrived fasting for the experiments, which were carried out from 9–12 a m

Two of those (B and P) received on two separate days 1) 400 mg of theophylline dissolved in 75 ml water and 2) 400 mg of theophylline dissolved in 75 ml 20% alcohol Only B consented to undergo an additional experiment, together with three other persons (K, F and N), in which 2 ml of *injectabile theophylamini fortius**) were given

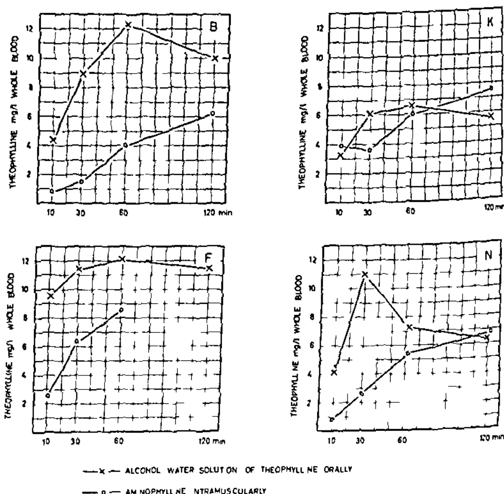


Fig 1 Blood theophylline levels following equivalent amounts of alcohol water solution of theophylline orally and aminophyllin intramuscularly

*) Injectabile theophylamini fortius is a sterile solution of theophylamine (Ph Dan) This solution contains in 1 ml 200 mg theophylline and 55 mg ethylene diamine hydrate

Acta pharmacol et toxicol 1961, 18 133–140

From the Department of Physiology University of Turku Finland
(Professor Kaarlo Hartiala M D)

The Absorption and Excretion of Phenolphthalein and its Glucuronide by the Cat.

By

Kalle Pekanmaki and Heikki A Salmi

(Received February 22 1961)

The glucuronidogenic characteristics of phenolphthalein were demon

[illegible] experiments on the cat have shown the same thing (PEKANMAKI 1958) The absorption of phenolphthalein from the digestive tract occurs to a negligible extent as the free compound It has been claimed that the absorption takes place as a protein complex (FANTUS & DYNIEWICZ 1937) The absorption of the pure conjugate phenolphthalein glucuronide has not been studied

The hepatotropic property of phenolphthalein and chemically related compounds demonstrated previously has been exploited for X ray examinations Tetra iodo- and tetrabromophenolphthalein are commonly used as contrast media in cholecystography Phenolphthalein in the free form is water soluble only at alkaline reactions In this respect it resembles bilirubin another compound also forming glucuronide complexes and soluble in bile

Studies with C^{14} labelled phenolphthalein (VISEK LIU & ROTH 1956) have indicated that the *molecule* of phenolphthalein is preserved intact when *introduced into the organism the authors* demonstrated the absence of radioactive CO_2 *from* the expired air [illegible]

[illegible] workers used the dog as the test animal and showed that the excretion takes place in both free and conjugated forms

of theophylline was as rapid from the aqueous as from the aqueous alcohol solution The maximum concentration occurred as early as 10–30 minutes after intake The concentration curve for alcohol shows a steeper rise to maximum values than that for theophylline

The two volunteers who drank the solution of 400 mg theophylline in water suffered from severe lower abdominal colics, accompanied by nausea, 6–8 hours after the intake Both had to lie down for almost an hour, and the symptoms did not disappear until the next day On no occasion did the aqueous alcohol solution give rise to any discomfort We have subsequently adopted the aqueous alcohol solution in treatment of acute asthmatic attacks in 16 patients and have so far observed no side effects of the kind described above

Conclusion and Summary.

For practical reasons oral intake of theophylline is preferable to injections SCHLUGER, MCGINN & HENNESY (1957) showed that the absorption of theophylline from an aqueous alcoholic solution was quicker than from tablets Our own experiments have shown a rate of absorption from this solution equal to, or even greater than, the absorption after intramuscular injection We have been unable to demonstrate any advantageous effect of adding alcohol on the rate of absorption It seems, however, that alcohol prevents the side effects on the gastro-intestinal tract that may arise after oral intake of 400 mg theophylline in aqueous solution

Acknowledgments

We thank dr J A Larsen, Dep of Physiology, University of Copenhagen, for the alcohol determinations We are indebted to the Arvid Nilsson foundation for financial support

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Phenolphthalein Determination in Bile

The bile specimen was hydrolysed overnight at 37 0°C in 0 1 M acetate buffer pH 4 5 containing 1 mg/1 ml β-glucuronidase (Worthington) The hydrolysate was extracted twice with ethyl ether in presence of 0 1 ml 1 N HCl, and the ethereal phase was separated The ether was evaporated in the water bath and the residue dissolved in glycine and sodium hydroxide buffer at pH 10 45 This solution was filtered with double filter paper (SCHLEICHER & SCHÜLL 589³) The absorbency of the liberated phenolphthalein was measured in a Beckman DU spectrophotometer at wavelength 540 mμ.

The concentration of free phenolphthalein was measured by a similar procedure, the β-glucuronidase hydrolysis being omitted

The amount of phenolphthalein was high enough in all experiments for the traces of bile pigments not to interfere with the determination

Phenolphthalein Determination in Blood

The proteins of whole blood were precipitated by acetone 10 part per 1 parts of blood The acetone blood mixture was centrifugated and the clear liquid evaporated in a warm water bath The residue was dissolved in 0 1 M acetate buffer pH 4 5 containing 1 mg/1 ...
37° Glycine-NaOH ...
filter paper (SCHLEI...
filter, the operation ...

The colour was measured as described above The concentration of free phenolphthalein was measured by a similar procedure, but without the β-glucuronidase hydrolysis

The ...
the β-glu...
were st... ...the action of urinary enzymes

Chromatographic Methods

For chromatographic analysis the conjugated phenolphthalein was extracted from bile and urine with ethyl acetate in acid medium

A descending method on Whatman No 1 paper and with butanol-ammonium hydroxide eluant was employed Glucuronides were hydrolysed on the paper with β-glucuronidase solution The liberated phenolphthalein was detected with glycine and sodium hydroxide buffer

The control substances were phenolphthalein mono-β-glucuronic acid (Sigma), phenolphthaleindisulphate (Sigma) and phenolphthalein pro anal (Merck)

Results.

The effect of bile on the absorption of phenolphthalein and its glucuronide is shown in fig 1 and 2 Some of the results were reported in a previous communication (PEKANMAKI & SALMI 1960)

Radio-isotope studies have indicated that, irrespective of whether phenolphthalein was given to dogs orally or intravenously, little more than a half of the radioactivity was excreted in the faeces. Of radioactive phenolphthalein given intravenously 43% was excreted into the bile over 72 hours, 11% in the faeces and 35% in the urine (VISEK, LIU & ROTH 1956) Investigators have not paid much attention to the form in which phenolphthalein is excreted, namely whether free or conjugated.

Demonstration of the cat's glucuronide-forming capacity in its intestinal tract (PEKANMÄKI 1958) has led us to the attempts, described here, to study the absorption and excretion of phenolphthalein and its glucuronide into the bile and urine.

Materials and Methods.

Altogether 13 female cats were used in the experiments

1) Phenolphthalein (Merck, pro analysi) and 2) phenolphthalein-mono-β-glucuronic acid (Sigma) were given to the animals a) into the stomach by means of a rubber tube, b) by injection into the portal vein, c) by injection into the caval vein Phenolphthalein solution was prepared by dissolving it in 2 N-NaOH and adding distilled water to give a final concentration of 0 02 N-NaOH Phenolphthalein mono-β- glucuronic acid was dissolved in distilled water

The materials analysed after various intervals were blood from the portal vein, blood from the caval vein, bile and urine

Bile and urine were collected quantitatively during the experiments Two different methods for collecting the bile and preventing drainage into the intestine were employed First, the common bile duct was ligated and the hepatic duct cannulated with a polythene tube to allow drainage of the bile from the liver through the cannula and prevent the flow into the bladder and intestine This operation was performed the day before the experiment proper and resulted in a total absence of bile from the intestine Second, the common bile duct was ligated and the gall bladder was evacuated at the beginning of the experiment proper by puncturing the wall. During the experiment the excreted bile was quantitatively obtained at the desired intervals by injecting saline into the bladder and sucking out the fluid several times, thus washing the inner walls of the bladder The operations and the experiments themselves were performed under pentobarbital anaesthesia. In the oral experiments, to prevent vomiting chlorpromazine at a dose of 4 mg/kg of body-weight was given half an hour before the beginning of the experiment.

The urine specimens were taken by collecting all urine excreted during the experiment and rinsing the bladder with distilled water at the end of the experiment in the same way as described in connection with the bile collection

The relative short duration of the experiments arose from the need to performance of somewhat drastic surgical operations Prolongation of the experiments induced in the animals disturbances of acidbase and mineral equilibrium.

Analytical Methods

Ether extraction was conducted by the method of FANTL & DYNIEWICZ (1937) Controls indicated that the β-glucuronidase hydrolysis described here does not split phenolphthalein sulphate

Phenolphthalein Determination in Bile

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The chromatographic analysis revealed the conjugation of phenolphthalein into the glucuronide in the bile By the descending chromatographic method the same value of *Rf* was obtained for the conjugate found in the bile as for the phenolphthalein mono β-glucuronic acid used as control ($Rf = 0\ 19$) After hydrolysis with β glucuronidase free phenolphthalein was detectable on the paper The same results were obtained on analysing urine

When the bile excretion of phenolphthalein and phenolphthalein glucuronide, given intravenously or orally, was studied as a function of

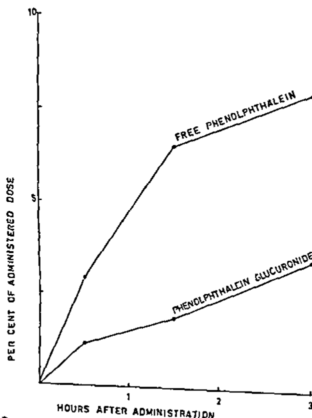


Fig 3 Excretion of phenolphthalein as glucuronide into the bile after oral administration The total excretion after oral administration of free phenolphthalein or phenolphthalein glucuronide Dosage 6 64 mg/kg body weight, expressed as phenolphthalein

time, greater excretion of free phenolphthalein was established at the same concentrations (Fig 3 and 4)

When the total amounts of absorbed phenolphthalein and phenolphthalein glucuronide were determined, it was established that the degree of absorption depended largely on the amount administered The per

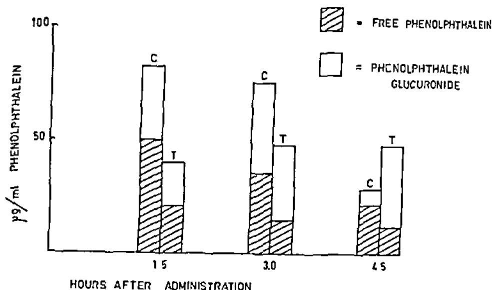


Fig 1 Effect of bile on absorption of phenolphthalein Concentrations in portal vein blood after oral administration of free phenolphthalein (100 mg/kg of body weight) T = test animals (ligation of common bile duct and drainage of bile) C = control animals (no ligation)

A clear decrease in blood concentration of free phenolphthalein was observed when drainage of bile into the intestine is prevented the concentration of phenolphthalein glucuronide in the blood remained almost unchanged

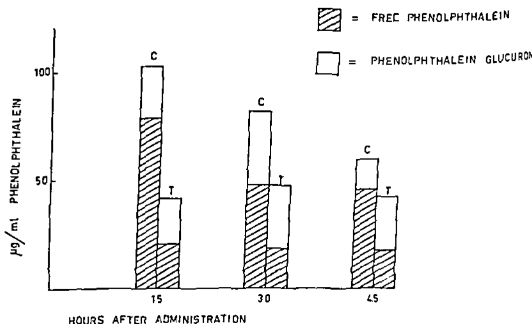


Fig 2 Effect of bile on absorption of phenolphthalein Concentrations in the vena cava blood after oral administration of free phenolphthalein (100 mg/kg of body weight) T = test animals (ligation of common bile duct and drainage of bile) C = control animals (no ligation)

When phenolphthalein glucuronide was injected into the portal vein, about 20% was detected in the bile and about 6% in the urine over 3 hours The corresponding values for phenolphthalein glucuronide given into the caval vein were 6% in the bile and 4% in the urine A summary of these results is shown in table 1

Table 1

Excretion of Phenolphthalein and its Glucuronide

Site of application	Substance administered							
	Phenolphthalein glucuronide				Free phenolphthalein			
	Sample: Bile		Sample: Urine		Sample: Bile		Sample: Urine	
	G	F	G	F	G	F	G	F
Digestive tract	+	±	+++	+	+++	±	++	++
Portal vein	++++	±	+++	+	±	±	+	++++
Vena cava	+++	±	++	+	++++	±	+	++

G = phenolphthalein glucuronide
F = free phenolphthalein
+ = < 2%
++ = 2–6%
+++ = 6–10%
++++ = > 10%
(per cent of administered dose)

Discussion.

The absence of the bile from the intestine has an influence on the absorption of *free phenolphthalein*, but not on the absorption of phenolphthalein glucuronide owing to the solubilising effect of bile salts and acids This possibility was suggested by FANTUS & DYNIEWICZ (1937) Thus glucuronide conjugation taking place in the intestinal mucosa plays an important part [illegible]

W[illegible]

phth[illegible] the bile is conjugated *solely with glucuronic acid* We have not studied the possible *structural changes* of phenolphthalein glucuronide during the *absorption stage, but it is certainly* absorbed unchanged from the digestive tract *and easily* excreted through the kidneys after entering the circulation On the other hand, phenolphthalein glucuronide administered *directly into the blood* is more easily *excreted* into the bile than into the urine *Free phenol*phthalein is still more actively excreted into the bile at the *same concentrations* Owing to the molecular structure *of phenolphthalein it is possible* that the free -OH radicals of a glucuronidogenic substance *are a necessary* condition for excretion into the bile It seems clear that *we are* dealing with an active *transport* mechanism

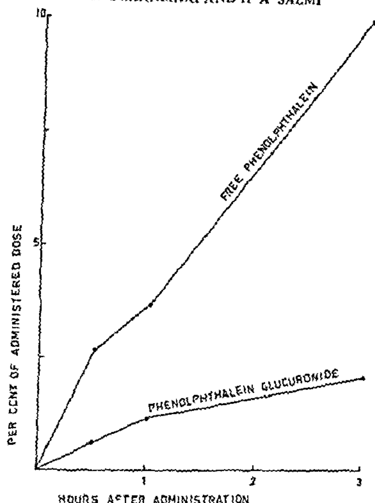


Fig 4 Excretion of phenolphthalein as glucuronide into the bile after vena cava administration Total excretion after oral administration of free phenolphthalein or phenolphthalein glucuronide Dosage 6 64 mg/kg body weight, expressed as phenolphthalein

cent absorptions of free phenolphthalein was greatly raised when the dosage was lowered The percentage of absorbed phenolphthalein ranged from 0 5–21, according to the dose given When free phenolphthalein was administered into the intestinal tract, the total amount excreted into the bile was about three times that excreted into the urine When phenolphthalein glucuronide was given by mouth, lowering the dose raised the percentage absorbed The percentage of absorbed phenolphthalein glucuronide was from 3% to 11% during 3 hours and always greater than the percentage of free phenolphthalein absorbed

When phenolphthalein glucuronide was given into the intestine, excretion into the urine was greater than into the bile

When phenolphthalein was injected intravenously it was established that 17% of the drug injected into the vena cava was excreted in 3 hours into the bile and 2% into the urine After administration by portal vein, the greater part of the phenolphthalein in the urine was in the free form

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Pharmacological Effects of Fatty Acids, Triolein and Cottonseed Oil.

By

Lars Oro and Arvid Wretlind

(Received March 8, 1961)

Various triglycerides in emulsion show marked pharmacological properties on intravenous administration For example, several with low molecular weights have been found distinctly toxic on intravenous injection (WRETLIND 1957 b, ORÖ, WESTERHOLM & WRETLIND 1959) On the other hand, fats of higher molecular weights, such as cottonseed oil, triolein and certain vegetable fats, show little or no toxicity on intravenous injection into mice Hence it is possible to produce injectable emulsions of cottonseed oil as a carrier of agents, such as higher fatty acids, that owing to insolubility could not otherwise be studied for their pharmacological effects Such fatty acid emulsions have enabled us to determine the toxicity of fatty acids on intravenous injection

In the same investigation we have also studied the effects of these emulsions on respiration and blood pressure in cats It was found that emulsions containing only cottonseed oil or triolein could cause reduced blood pressure and apnoea These effects were characterised by such pronounced tachyphylaxis that frequently they were noticeable only on the first injection They were readily distinguishable, therefore, from the affects of the different fatty acid emulsions in which cottonseed oil acted as carrier

Lastly, we conducted experiments on guinea pig small intestine in order to study the effects of fatty acid emulsions on smooth muscle

Experimental.

Animal Experiments

For *determinations of toxicity*, mic[illegible]
solutions of fatty acids were injected [illegible]
to at least six groups each of 10 anim[illegible]

The free form of phenolphthalein was absent from the bile, but the small amount of material precludes the drawing of too dogmatic conclusions from this finding

Summary.

The absence of the bile from the intestine reduced the absorption of free phenolphthalein, but had no influence on the amount of absorbed phenolphthalein glucuronide when conjugated phenolphthalein was excreted into the bile in the form of glucuronide only

No free phenolphthalein was detected in the bile Of administered free and conjugated phenolphthalein the free form was excreted more intensively

Phenolphthalein glucuronide was excreted into the urine more easily than the free form

The degree of absorption of free and conjugated phenolphthalein depended largely on the amount administered The percentage absorption rose when the dosage was lowered

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Table 1

Composition of fatty acid emulsions used for determining the toxicity in mice

[illegible] Snow [illegible] s used [illegible] re pre [illegible] ulsions

Fatty acid	0 2–2 g
Cottonseed oil	10 g
Phosphatides	0 4 g
Sodium cholate	0 1 g
Glycerol monostearate	0 5 g
5% glucose solution to	100 ml

higher fatty acids than the emulsifiers previously mentioned The fatty acids investigated were stearic, oleic, lauric and tridecylic

The *cottonseed oil emulsions* used in the cat experiments had the compositons shown in table 2, except that they contained no fatty acid and that either unsaturated phosphatides (WRETLIND 1957 a) or hydrogenated phosphatides were used In some experiments we studied emulsions containing triolein instead of cottonseed oil

The *hydrogenated phosphatides* were prepared as described below The phosphatides 320 g were dissolved in 600 ml cyclohexane 3,400 ml absolute alcohol were added, and the precipitated cephalins were filtered off The filtrate was concentrated under reduced pressure and nitrogen to a volume of 850 ml To the resulting solution were added 30 mg Raney nickel as catalyst the solution being then transferred to an autoclave in which it was subjected to hydrogenation at 85–130°C with hydrogen at a pressure of 75–150 atmospheres for two hours The hydrogenated solution was filtered hot and passed through a column of 500 g Al_2O_3 with a diameter of 45 mm, equipped with a heating jacket (75°) The column was eluted with 95% ethanol The first fraction of 300 ml was discarded, the next two litres of eluate were added to six litres off acetone and placed in a refrigerator at +4° The resulting phosphatides were filtered of under reduced pressure washed with acetone, and dried in a desiccator under reduced pressure The yield amounted to 30–50 grams Analysis showed N 1 6–1 7 per cent and P 3 7–3 8 per cent

Table 2

Composition of emulsions used for investigating the effects of fatty acids on respiration and blood pressure

Pluronic F 68 (polypropylene polyethylene glucose) obtained from Wyandotte Chemical Corp, Wyandotte, Ill U S A The emulsions were adjusted to pH 7 3 with 1 N NaOH

Fatty acid	1 g
Cottonseed oil	10 g
Phosphatides	1 2 g
Pluronic	0 4 g
5% glucose solution to	100 ml

errors were by the method of MILLER & TAINTER (1944), the results are summarised in figure 1

Some 100 cats weighing 2-6 kg were used for investigating the *respiratory* and *circulatory responses to cottonseed oil, triolein* and *fatty acid emulsions* These animals were anaesthetized with diallymal (allobarbital (WHO) dial ®, Ciba, 25-40 mg per kg body weight) or chloralose (50 mg/kg) Respiration and blood pressure were recorded by means of a Grass electroencephalograph[1]) with supplementary demodulators A Statham pressure transducer, model P 23 AA[2]), was used for blood-pressure determinations on the carotid artery The respiration was recorded with a Grass pressure transducer PT-5 connected to a side tube in a tracheal cannula

In a number of experiments the *pulmonary arterial pressure* was measured via a plastic catheter that had been sutured in the artery after opening the thorax In one experiment the thorax was closed again, in the others it was left open under artificial respiration

For studying the direct effect of fatty acids upon the heart, a *heart lung preparation* described by WRETLIND (1957 b) was used Blood pressure and flow were recorded by the Grass transducers mentioned above

The action of fatty acids on guinea pig *small intestine* was studied in an isolated organ bath containing 15 ml Tyrode solution (0 8 %NaCl, 0 02% KCl, 0 02%$CaCl_2$, 0 02% $MgCl_2$ 6 H_2O, 0 1% $NaHCO_3$, 0 005% NaH_2PO_4 H_2O, and 0 1% glucose) The organ bath was aerated with air A Grass force-displacement transducer, model FT-03, *was employed for recording the tension of the gut In this connection* we also studied the ability of fatty acids to abolish contractions elicited by histamine For this purpose histamine (0 2 μg/15 ml) was first added to the organ bath and fatty acid emulsion was added without prior washing when maximal contraction had been obtained The observed relaxation of the intestine was recorded

Test Solutions and Test Emulsions

For the *toxicity determinations* in mice the lower *fatty acids* (acetic, propionic, butyric, isovaleric, norvaleric, caproic and heptylic) were administered in aqueous solution, whereas the higher fatty acids, because of their insolubility in water, were emulsified To prepare these *emulsions* the fatty acids were first dissolved in heated cottonseed oil, which served as carrier Phosphatides, sodium cholate and glycerin monostearate were used as emulsifiers (table 1) The emulsions were homogenized in a Logeman apparatus (WRETLIND 1957 a) The pH of the solutions and emulsions was adjusted to 7 3 with NaOH

The concentrations of fatty acids were so chosen that the injected volume required for determining LD50 amounted to between 3 and 45 ml per kg body weight Thus acetic, propionic, butyric, isovaleric, norvaleric and caproic acids were administered as 10% and heptylic acid as 5% aqueous solutions, caprylic, nonanoic, capric, undecylic, lauric, tridecylic and myristic acids as 2% emulsions, pentadecylic, palmitic, margaric and stearic acids as 0 2%, and oleic acid as 2% emulsions

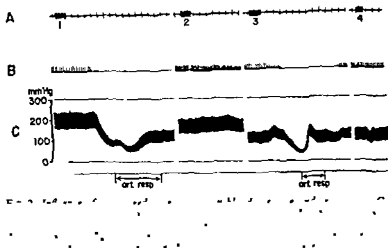
For investigating the action of fatty acids on *guinea pig gut* 1% emulsions of the *compositions recorded in table 1 were used*

Cottonseed oil was similarly utilized as carrier when investigating the *respiratory* and *blood pressure* responses of the cat to higher fatty acids Emulsification was with phosphatides and Pluronic F 68 (table 2), because they make stabler emulsions of

1) Grass Instrument Co , Quincy Mass , U S A
2) Statham Instruments Inc , 254 Carpenter Road, Hato Rey, Puerto Rico

stearic acid, which had an LD50 of 23 ± 0 7 mg per kg Approximately the same value was obtained for stearic acid in the rat (21 5 ± 1 8 mg/kg) Oleic acid showed an LD50 of 230 ± 18 mg/kg in mice This implies that stearic acid is ten times more toxic than the unsaturated oleic acid The toxicity of norvaleric and isovaleric acids differed slightly, LD50 being 1,290 ± 53 and 1,120 ± 30 mg/kg, respectively

When the fatty acid solutions and emulsions were injected in amounts near the LD50 dose, the animals immediately had convulsions and collapsed on their sides Respiration ceased within 1–2 minutes, sometimes after hyperpnoea



Intravenous administration of emulsion with *cottonseed oil or triolein alone to anaesthetized cats* produced various effects, depending on the dose and the emulsifiers With soy bean phosphatides and Pluronic F 68 as emulsifiers an initial intravenous injection (0 2–1 ml/kg body weight) sometimes caused a fall of blood pressure as well as apnoea (fig 2) The latency was usually around 10–40 seconds Another injection of the same dose invariably had either a less effect or none at all – *The action of these cottonseed oil emulsions is thus characterized by pronounced tachyphylaxis, hence the same or progressively higher doses can be repeated with no demonstrable effects on blood pressure and respiration* – When Pluronic F 68 alone was the emulsifier, 1–3 ml/kg was required to produce an effect with 10% cottonseed oil or triolein emulsion

Cottonseed oil emulsions produced a rise of pressure in the *pulmonary artery* up to 100 mm Hg (fig 3) The latency varied from 10 seconds to

Results.

In attempts to determine the LD50 for the carrier emulsions with *cottonseed oil alone*, we found on intravenous injection of a maximal volume – i.e., *60 ml/kg of the 25% cottonseed oil emulsion* with emulsifiers, as shown in table 1 – *into mice* only a transient depression of the respiration rate. It follows that the LD50 is higher than 15 g cottonseed oil per kilogram.

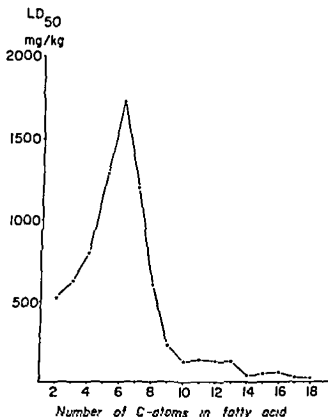
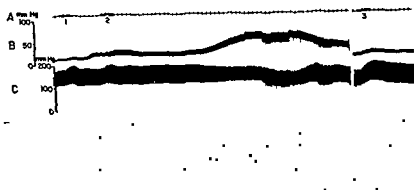


Fig. 1. *Toxicity of saturated fatty acids on intravenous injection into mice* The abscissa indicates the number of carbon atoms in the fatty acid molecule, the ordinate, LD50 in milligrams per kilogram of body weight The LD50 values in mg/kg, together with their standard errc ... butyric acid 800 ± 24; n-valeric ... 5 ± 85, heptylic acid 1200 ± ... acid 129 ± 5 4; undecylic aci ... 0, myristic acid 43 ± 2 6, pe ... acid 36 ± 0 3;

Figure 1 shows that the intravenous *toxicities of different fatty acids* differ considerably for mice. Least toxic is caproic acid with six carbon atoms, which has an LD50 of 1,725 ± 85 mg per kg body weight. With an increased or decreased number of carbon atoms in the fatty acid molecule, the toxicity rises. The most toxic of all those investigated was

the cottonseed oil, but on repeated injection these quickly subsided because of the tachyphylaxis With fatty acid emulsions containing hydrogenated phosphatides, only the effects of the fatty acids were observed

Intravenous injection of the *fatty acids* investigated – stearic, oleic, lauric and tridecylic – had a hypotensive effect on the systemic circulation, but raised the pulmonary arterial pressure The doses required for this action were 5 mg stearic acid, 50 mg oleic acid, 75 mg lauric acid or 60 mg tridecylic acid per kg body weight All of the fatty acids studied had a negatively inotropic effect on the heart, as shown by the experiments on heart lung preparations Similar results were given by oleic acid emulsions



without cottonseed oil as carrier and also by those with unsaturated phosphatides and Pluronic F 68 as emulsifiers Emulsions with different emulsifiers differed somewhat in their effects For instance, the effect on pulmonary arterial pressure was far less with hydrogenated than with unsaturated phosphatides The action on the *respiration* was characterized by hyperpnoea after small doses and apnoea after larger ones No tachyphylaxis was observed with fatty acid emulsions In several experiments indeed an increased effect was noted on repeated injection of equal doses (figures 6 and 7) – Vagotomy had no effect on falls in blood-pressure produced by the fatty acids studied

As to the action of *fatty acid emulsions on small intestine from the guinea pig* our results indicated that the emulsions did not themselves cause any contraction even in relatively large doses (100 mg/15 ml) They could however inhibit the contraction produced by histamine The degree of inhibition depended on the particular fatty acid contained in the emulsion Fig 8 shows the relative potencies of the fatty acids in this respect It will be seen from the curve that capric and undecylic acids, i e, those with 10 or 11 carbon atoms, were the most effective Somewhat more than 15 mg/15 ml of these acids was required for total inhibition of

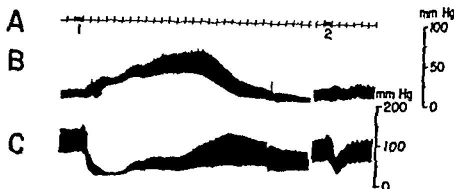


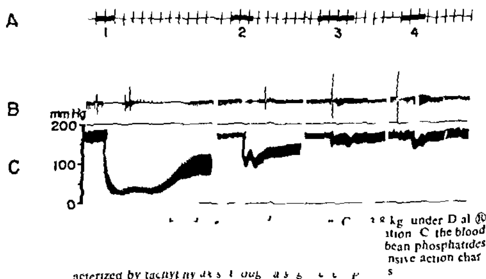
Fig 3 *Effect of cottonseed oil emulsion on pressure in the pulmonary and carot d arteries*
Cat ~ A ~ is
and
0 5
as emuls fiers were injected

2 *minutes* Here too the effect declined with repeated injections and ultimately disappeared, although the injected doses were high

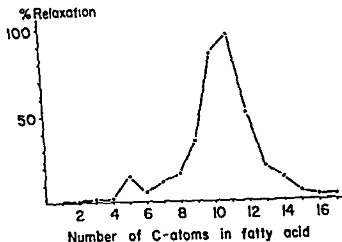
The hypotensive action of these emulsions was substantially less after vagotomy, but in spite of this operation there was an unchanged rise of pressure in the pulmonary artery This elevation in pressure showed a high degree of tachyphylaxis (fig 5)

Direct experiments showed also that soyabean phosphatides caused a blood pressure fall and apnoea both of which effects exhibited marked tachyphylaxis With repeated injections however the hypotensive action did not completely disappear (fig 4) a result suggesting that two hypo tensive factors were present here

With cottonseed emulsions containing fatty acid the first injections were sometimes accompanied by interference with the specific effects of



~ C 2 8 kg under D al ation C the blood bean phosphatides nsive action char acterized by tachyphylaxis



Discussion.

This investigation thus shows that several fatty acids are markedly toxic when administered intravenously It is mainly the higher fatty acids that show this (LD50 23 mg/kg) to the greatest extent With a reduced number of carbon atoms in the molecule the toxicity diminishes, being least for caproic acid with six carbon atoms (LD50 1,724 mg/kg) When compared with the triglycerides of low molecular weight, the free fatty acids thus show the reverse behavior in toxicity, for the LD50 curve for triglycerides reveals a distinct minimum when the fatty acid component has five or six carbon atoms Thus, hydrolysed fatty acids cannot be responsible for the acute toxic action of the triglycerides

Our value for the LD50 of acetic acid (525 mg/kg) is closely consistent with that found by WELCH *et al* (1944) – 380 mg sodium acetate per kg in mice SAMSON, DAHL & DAHL (1956) described a hypnotic action of fatty acids of low molecular weight (C_2–C_{10}) There seems to be no direct correlation between hypnotic and toxic effects The ED50 for hypnotic action decreases progressively with an increasing number of carbon atoms in the fatty acid molecule, in contrast to the LD50, the highest dose being that of caproic acid with six carbon atoms and the dose then falling with both increased and reduced numbers of carbon atoms

The responses of respiration and blood pressure to the fatty acid emulsions differed from those to the triglycerides of low molecular weight

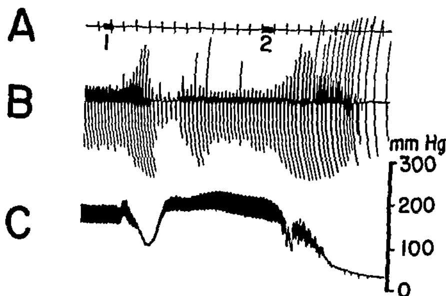


Fig 6 *Effect of oleic acid emulsion on blood pressure and respiration* Cat, 4 0 kg under chloralose anaesthesia with bilateral vagotom A nd ... jection times, B, resp ... emulsion in cottonsee

the contraction after 0 2 µg histamine If the fatty acid molecule contained a greater or smaller number of carbon atoms than 10 or 11, the relaxing action on histamine-contracted intestine decreased, for instance, 150 mg of butyric acid was required for total inhibition Comparison of undecylic acid with its unsaturated homologue, undecylenic acid, revealed no significant difference. The emulsion system alone without fatty acid is inert No difference was detectable when the lower fatty acids were administered in aqueous solutions instead of in emulsions

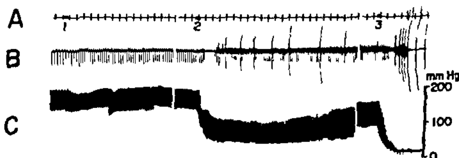


Fig 7 *Effect of stearic acid emulsion on respiration and blood pressure* Cat, 4 3 kg under

action of serotonin (5 hydroxytryptamine) and the effect in anaphylactic shock in mice

As to the blood pressure fall that follows injection of emulsions of *cottonseed oil* or *triolein* it is difficult to determine the mechanism exactly because of the pronounced tachyphylaxis

If an anaesthetized cat is injected with 6 ml/kg 10% emulsion of cottonseed oil or triolein emulsified with 0 1 or 1 0% Pluronic F 68 a substantial fall in blood pressure as well as apnoea occur after about 10–60 seconds The cat may even die after this first injection The pul monary arterial pressure sometimes rises as high as 100 mm Hg Our results suggest that the hypotensive effect is elicited mainly via the vagus for it is practically eliminated by vagotomy (fig 5)

Since the responses of blood pressure can also be obtained with the soya bean phosphatides alone it seems probable that the effects observed with the cottonseed oil and triolein emulsions were due to the presence of such phosphatides That the unsaturated phosphatides were responsible also appears likely inasmuch as there was no circulatory response when hydrogenated soya bean phosphatides were used as emulsifiers The fact that cottonseed oil emulsions with Pluronic F 68 as the sole emulsifier produced similar effects on the blood pressure shows that this is not the only explanation Our observations tend rather to suggest that the effects of intravenously administered fat emulsions are due to a number of fac tors such as the properties of the fat the emulsifiers the surface tension size of fat particles and the charge carried by them On the basis of our present knowledge in this field it is impossible to say finally which is the principal factor involved

According to SEEVERS (1958) tachyphylaxis is probably due to specific receptors that may take up the active substance thereby eliciting the pharmacological action When no more of these receptors are available the effect ceases The active substance is subsequently released slowly and not until the liberation is complete can an effect be once more ob tained In our experiments the recovery period was relatively long at least four hours It is not yet possible however to say where these receptors are situated or by what mechanism the fat emulsions block them

Summary

Pharmacological investigations of the effects of intravenously admin istered fatty acids of high molecular weight are handicapped by the fact that such acids are insoluble in water at neutral reaction By utilizing fat particles in an emulsion as carriers of these fatty acids it has been possible

(WRETLIND 1957 a), in cats intravenous injection of more than 5 mg stearic acid or 50 mg oleic acid per kg was followed by apnoea and a fall in blood pressure as well as by convulsions with lethal outcome Lower doses had a depressor action on blood pressure, the respiration sometimes being stimulated The dose required for toxic action varied somewhat with the emulsifier employed The effect on the circulatory system also *differed with different emulsifiers* Stearic acid emulsions with phosphatides and Pluronic as emulsifiers raised the pulmonary arterial pressure substantially, *and this elevation contributed to the fall of blood pressure* in the systemic circulation With hydrogenated phosphatides there was no demonstrable influence on pulmonary arterial pressure, on the other hand these emulsions had a pronounced effect on heart-lung preparations so that their hypotensive action may be attributable to direct cardiac depression Oleic acid emulsions that had been emulsified solely with phosphatides and Pluronic F68, without cottonseed oil, also had a hypotensive and toxic action on heart-lung preparations

It is accordingly evident that the emulsified higher fatty acids studied had, on intravenous injection, a pronounced toxic action associated with a fall of blood pressure

PELTIER *(1956) determined the toxicity of some neutral fats* and free fatty acids in non emulsified form He concluded that the toxic effects of fatty acid were caused by damage to the pulmonary capillaries with extravasation into the alveolar spaces JEFFERSON (1948) held a similar view In our experiments on anaesthetized cats we occasionally observed progressive respiratory distress, and blood tinged froth escaped from the tracheal cannula At autopsy the lungs were oedematous – It is worthy of note that the LD50 is more or less the same for non emulsified oleic acid in rabbits (PELTIER 1956) and emulsified oleic acid in mice (250 mg/kg *and 230/kg respectively)* SCUDERI *(1941) found that 0 33 ml of* non emulsified oleic acid per kg had a toxic action on the dog The emulsified form had a lethal effect on anaesthetized cats in somewhat smaller doses, i e, 50–100 mg/kg However, total amounts substantially greater than 250 mg of emulsified oleic acid per kg can be given by repeatedly injecting sublethal doses

The fatty acid emulsions had no stimulating action on *small intestine from the guinea pig* – a finding that accorded with results reported by GABR (1956) The emulsions could however, relax histamine contracted intestine *This effect was not correlated with their toxic action*, rather the reverse, the relaxing effect being maximal for fatty acids with 10 or 11 carbon atoms and decreasing with either a smaller or greater number of carbon atoms in the molecule Of interest in this connection is the finding of GANLEY *et al* (1960) that higher fatty acids partially inhibited the

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From the Department of Pharmacology, University of Gothenburg, Sweden
(Prof N-O Abdon M D †)

The Effect of a Surface-Active Agent ("Tween 20") on Speed of Transportation by the Frog's Ciliated Mucous Membrane.

By

Carl Ryde

(Received March 14 1961)

Ciliary function is of a great importance for the forward movement of mucus along the bronchial mucous membrane Several investigations have been carried out on the effect of various drugs on the ciliary mechanism, especially those used as expectorants (KOCHMANN 1930, RICHTNÉR 1941; KORDIK *et al* 1952, HILL 1957) It has been demonstrated that, among other compounds, ammonium chloride, iodine compounds, ethereal oils and saponins stimulate ciliary movement, whereas morphine inhibits it

LIESEGANG (1939) emphasized the importance of the pure physical factors that come into play with expectoration, he was of opinion that the agents reducing surface tension would facilitate expectoration through this action

During recent years surface active agents have been used in increasing degree, and with good clinical results, for the treatment of coughs

MILLER & BOYER (1952) used "W R 1939", an alkylarylpolyether alcohol, and LARKIN (1957) polyoxyethylenedodecanole, each *given by* inhalation MILLER was of the opinion that the favourable clinical effect occurred because the surface active agent lowered the viscosity of the sputum FORBES & WISE (1957), however, in similar experiments, found no appreciable change in viscosity

RYDE (1955) tested polyoxyethylene sorbitan monolaurate (Tween 20) by mouth It has been shown that a syrup containing this agent (Expigen ®) had a marked "loosening up" effect, especially in patients with troublesome coughs and viscous expectorations

The difference between Tween 20 ® and Tween 80 ® (= sorbimacrogol oleate 300, WHO NFN) is that the fatty acid component [illegible]
saturated whereas Tw[illegible]
with the WHO and N[illegible]
"sorbimacrogol laurate [illegible]

in our investigation to study the toxicity and certain pharmacological effects of such acids when given intravenously Although emulsions of the carrier fat are devoid of toxicity, they exhibit peculiar pharmacological effects characterized by pronounced tachyphylaxis In this investigation the following observations were made

The LD50 by intravenous injection into mice was determined for *saturated fatty acids* from C_2 to C_{18} Of these, stearic acid emulsion showed the highest toxicity LD50 23 mg/kg body weight With a decreasing number of carbon atoms in the fatty acid molecule the toxicity fell reaching a minimum for caproic acid LD50 1,725 mg/kg As the number of carbon atoms declined beyond that point, so did the toxicity rise again the LD50 amounting to 525 mg/kg for acetic acid

The LD50 for stearic acid was ten times less than for its unsaturated analogue, oleic acid For the *cottonseed oil emulsion* employed as "carrier the LD50 was indeterminable, being higher than 15 g cottonseed oil per kilogram

The pharmacological effects of emulsions of *cottonseed oil and triolein* on respiration and blood pressure were investigated by intravenous injection into cats With soya bean phosphatides and Pluronic F 68 as emulsifiers a blood pressure fall and apnoea as well as an elevation of the pulmonary arterial pressure were observed These effects were also noted for fat emulsions emulsified solely with Pluronic F 68 and for unsaturated soya bean phosphatides alone Emulsions with hydrogenated phosphatides, on the contrary, did not produce such effects The action on the circulation and respiration showed marked tachyphylaxis

The effects of higher *fatty acid emulsions on circulation and respiration* were characterized by a fall in blood pressure as well as hyperpnoea or apnoea Such emulsions also had a negatively inotropic effect on the heart

Fatty acid emulsions had no direct action on *small intestine* from the guinea pig but inhibited contractions elicited by histamine

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The difference between the averages, tested by the t test, is highly significant (t = 12 7)

In the other test series, which consisted of 48 observations, a small piece of cork was used instead of a mucous clot, but under otherwise similar conditions The average values of these observations are given in table 2

Table 2

Transportation time in seconds of a piece of cork over a frog s ciliated mucous membrane moistened with isotonic sal ne solution with or without Tween 20 s e m standard error of mean

Solution applied	Time in sec	
	mean	s e m
0 6% saline	28 4	0 50
0 03% Tween 20 in 0 6% saline	27 4	0 52

The difference between these values is not significant (t = 0 9)

These results show that, with a mucous clot, there is a definite increase in transportation speed brought about by the presence of a surface active agent On the other hand, there is no effect on the transportation of a piece of cork

A change of transportation speed in a test such as this must depend either on a change in ciliary activity or on a change in the adhesive conditions

That the transportation speed increases only for an object showing marked adhesion to the mucous membrane (such as mucus) could be interpreted to mean that the surface active agent acts by diminishing the adhesiveness This explain the beneficial effect on expectoration that has been observed with cough medicines containing surface active agents as the active ingredient

Summary.

The effect of a surface active agent (polyoxyethylene sorbitan monolaurate Tween 20) on the transportation speed of ciliary mucous membranes from the frog has been investigated It was demonstrated that it caused a significant increase in speed of transportation of a mucous clot, but there was no observable change in speed of transportation of a piece of cork This may be due to the reduced adhesiveness caused by the surface active agent and might explain the beneficial effect of such agents on expectoration

Our own investigation was intended to discover whether or not "Tween 20" effects the speed with which a ciliary mucous membrane can transport particles placed on it

Methods.

The investigation was done on the pharyngeal mucous membrane of the frog (*Rana esculenta*) The frogs were of equal size and were judged to be all of equal activity They were kept at a controlled constant temperature, and the experiments were carried out at room temperature (20°C)

The animals were sacrificed by pithing through an incision in the neck, after which the brain and spinal cord were destroyed The ciliary mucous membrane in the pharynx and oesophagus was dissected out and fastened with clips to a glass slide The slide was then placed in a damp Petri dish containing a filter paper moistened with a 0 6% saline solution The preparation was examined under an operation microscope with a visual field of 13 mm, two series of tests were made The frogs in each series were divided into two groups picked at random In the first series the frogs in one group were treated with a drop of solution containing 0 03% Tween 20" in 0 6% saline, whereas the preparations in the other group received only a drop of 0 6% saline During each dissection, the thick mucus, always readily available on the mucous membrane, was collected Each frog s own viscous mucus was placed on the mucous membrane The amount was approximately constant The time was measured with a stop watch from the moment that a definite point on the mucous clot passed in and out of view In the second series, a diminutive piece of cork (a little under a mm in diameter) was used instead of the mucus The averages of 10 measurements on each preparation were determined

Results and Discussion.

In all 72 observations were made on the transportation time of a mucous clot In half the experiments, the mucous membrane was moistened with saline solution, in the other half with "Tween" solution The average values for the observations are given in table 1

Table 1

Transportation time in seconds of a mucous clot over a frog s ciliated mucous membrane moistened with isotonic saline solution with or without Tween 20
s e m = standard error of mean

Solution applied	Time in sec	
	mean	s e m
0 6% saline	32 09	0 43
0 03% Tween 20 in 0 6% saline	25 33	0 34

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From the Pharmacological Laboratory of Dumex LTD, Copenhagen
(E Jacobsen, M D)

Absorption and Excretion of Noscapine

By

Sven Vedsø

(Received March 28, 1961)

The clinical and biological effects of opium alkaloids have been known for a long time, but little has been published on their absorption and excretion Most of them are toxic even in small doses; for this reason methods for determining their concentrations in the body, for example in the blood, must be extremely sensitive if they are to give useful information

Opium contains 4 to 10 percent noscapine For many years it was assumed that this compound potentiated the effect of morphine COOPER & HATCHER (1934) disproved this conclusively and at the same time measured in cats the blood concentration of noscapine and its excretion via the urine However, their analytical methods were of low sensitivity, so that they could only state that the compound disappeared rapidly from the circulating blood after intravenous administration and that mere traces were found in the urine In recent years interest in noscapine as a therapeutic agent has been revived on account of the discovery that it has a pronounced antitussive effect (For references, see VEDSØ 1961) However it should be remarked that noscapine was recommended already as early as in 1930 by CHOPRA MUKHERJEE & DIKSHIT for being "by itself beneficial in allaying cough"

By a fluorimetric method described earlier (VEDSØ 1961) the concentration of noscapine in blood and urine has been investigated after oral administration to human subjects and after intravenous administration to rabbits

Methods.

Noscapine was given orally, in various doses, as tablets containing to normal male experimental subjects

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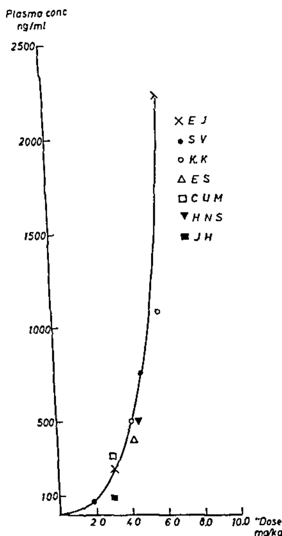


Fig 2 Relationship between dose of noscapine chloride and plasma concentration 2 hours after intake of noscapine chloride The values are taken from fig 1 and from 4 similar experiments

Abscissa Dose of noscapine chloride in mg/kg body weight
Ordinate Plasma concentration (ng/ml) 2 hours after administration

one maximal blood concentration was reached within 1 hour and then fell rapidly

The values in fig 2, taken from fig 1 and from 4 similar experiments, show the relation between the plasma concentration two hours after ·en, in mg per kg body weight The relation is not

The tablets were taken in the morning about two hours after a light continental breakfast Immediately beforehand a blood sample was taken At various times after intake (usually 1, 2, 3, 4, and 6 hours) blood samples were withdrawn and their contents of noscapine chloride determined All blood samples were taken from the cubital vein and stabilized with heparin

Some experiments were also carried out by oral administration of the same doses formulated as delayed absorption preparations Two techniques were used 1) every tenth minute the experimental subject was given 5 0 ml of a solution of noscapine chloride in sucrose syrup (3 2 mg/ml) 2) the experimental subject was given the noscapine as a cation-exchange resin complex of sulphonated polystyrene

In the animal experiments unanaesthetised rabbits were used and noscapine chloride was given in an isotonic solution intravenously into the ear vein The blood samples were taken at various times from the opposite ear

Results.

Plasma In fig 1 are shown the plasma concentrations obtained in 6 different experimental subjects all of whom took noscapine chloride in doses of 250–300 mg at zero hour and in one instance 500 mg In all but

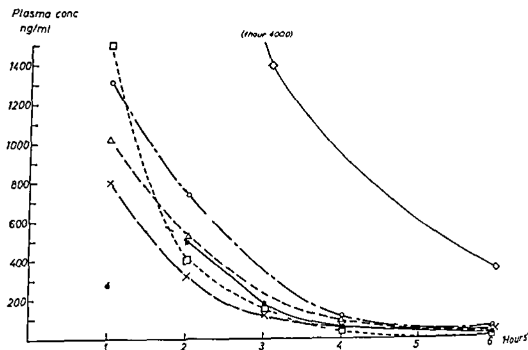


Fig 1 Concentration of noscapine chloride in the plasma of 6 human subjects given noscapine chloride (250–300 mg and in a single case 500 mg) orally at zero time

Abscissa Time in hours after administration
Ordinate Concentration (ng/ml) in the plasma

◇ ——— ◇ E J dose 500 mg
□ - - - - - □ E S – 300 mg
○ - - - - - ○ S V – 250 mg
△ - - - - △ K K dose 250 mg
× - - - - - × C M – 300 mg
● - - - - - ● H S – 300 mg

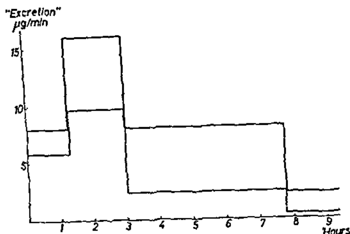


Fig 4 Excretion in the urine of free and total noscapine chloride after oral administration of 500 mg noscapine chloride

— — free noscapine
——— total noscapine

Abscissa Hours after administration
Ordinate Excretion in the urine in µg/min

different days This method of administering the alkaloid produces a steep fall in plasma concentration

Urine Noscapine is excreted in the urine partly free and partly conjugated as are morphine and many other alkaloids Determination of total noscapine was made after hydrolysis in 1 N HCl at 100° for 1 hour Fig 4 shows the relationship between free and total noscapine in urine at various intervals after the intake of noscapine chloride of a single experimental subject The excretion in the urine increased with the plasma concentration, individual variations were high, but only in two subjects out of 16 was concentration in the urine more than four times that in

after intravenous injection of noscapine chloride
the total amount of plasma, taken as 100 ml

after injection			
6–7 min	13 14 min	16–18 min	60–62 min
		0 350 µg/ml 0 7%	0 185 µg/ml 0 35%
8 5 µg ml 3 4%		4 5 µg/ml 1 8%	0 9 µg/ml 0 36%
	5 0 µg/ml 2.0%		

"Plasma conc"
ng/ml

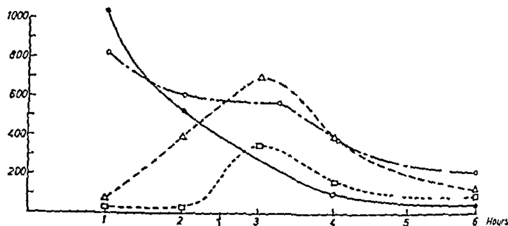


Fig 3 Comparison between plasma levels obtained after administration of different forms of the drug to the same experimental subject

●———● one single dose of noscapine chloride (250 mg)
△ - - - - - △ divided administration of noscapine chloride (250 mg) the arrow indicates last administration
□ - - - - - □ one single dose of noscapine resin complex (corresponding to 250 mg noscapine chloride)
○—— ——○ one single dose of noscapine resin complex (corresponding to 350 mg noscapine chloride)

Abscissa Time in hours
Ordinate Concentration (ng/ml) in the plasma

rectilinear but hyperbolic, so that the increase in blood concentration is relatively much higher than the corresponding increase in dose

Fig 3 shows a typical example of how the plasma concentrations obtained in the same person depend on the method used to administer the noscapine, as a single dose of chloride or in divided doses as chloride or as a cation exchange resin complex

Table 1 shows the plasma concentrations in 2 rabbits One received 5 mg and the second 25 mg noscapine chloride intravenously on two

Table I

Concentration of noscapine (as chloride) in rabbit plasma
1) Estimated percentage of given dose present in

Dose	Date	Rabbit	Minutes	
			2 3 min	2 5 min
5 mg	12/9	I 3 5 kg		2 75 µg/ml 5 5%1)
25 mg	14/9	II 3 15 kg	14 3 µg/ml 5 7%	
25 mg	17/9	II 3 15 kg	15 2 µg/ml 6 1%	

on it increased continuously during the period of administration, so that 3 hours after the first administration of the fractionated dose the plasma concentration was nearly 3 times as high as when the alkaloid had been administered as a single dose at zero time It is also seen (fig 3) that the concentration in the blood reached its maximum as early as about 30 minutes after administration had ceased

These results indicate that noscapine is not broken down rapidly, but that apparently, like morphine (MILTHERS 1958) and several other weak organic bases, such as certain antihistamines and antimalarials, it is deposited in the tissues, as described in reviews by BRODIE & HOGBEN (1957) and WAY & ADLER (1960)

The experimental subjects were only slightly troubled by the rather high doses of the alkaloid, of which the maximum single dose according to the Danish Pharmacopoeia is 50 mg The subjective symptoms after administering the alkaloid were not proportional to the blood concentration Some of the subjects felt slight drowsiness and in a single subject nausea was observed These symptoms, however, occurred as much as 2–3 hours after the intake of noscapine chloride, at a time when the plasma concentration was much lower than after 1 hour It does not therefore appear that any conclusion can be drawn from the plasma concentration as to the pharmacodynamic effect of noscapine On the other hand measurements of the plasma concentration can be used as an index of absorption rate During the absorption of a large amount in a short time from the intestinal tract as occurs after large doses of noscapine, the rate of fixation to the tissues will be slower than the rate of absorption As a result, the plasma concentration will rise When the doses are further increased, the disproportion between absorption and fixation rates becomes even more pronounced, and the peculiar hyperbolic shape of the dose plasma concentration curve, as seen in fig 2, appears The shape of this curve suggests that the noscapine in blood can be regarded as the result of a kind of overflow The results obtained with divided intake of the alkaloid support this view The curves obtained after fractional intake of the chloride were similar to those obtained in the experiments in which the alkaloid was administered as a cation-exchange resin complex, but apparently the absorption is prolonged with the last mentioned to 3–5 hours as seen from fig 3

Excretion of free noscapine in the urine increases with the plasma concentration However, the concentrations measured in the urine are generally only 2–4 times as high as the corresponding concentrations in the plasma Whether the small amount excreted is due to fixation by the plasma proteins or to some other process has not been investigated Initially, free noscapine is excreted almost exclusively, but later the

the plasma, no further investigation has been made into this point, and we have ignored the relation between free and conjugated noscapine in both plasma and blood Moreover the amount bound to plasma proteins is completely unknown

Discussion.

In man plasma concentrations of noscapine chloride obtained after each of the 3 methods of administration were small compared with the dose given In 5 out of 6 experiments (fig 1) the peak plasma concentration after a single oral dose noscapine chloride was obtained after 1 hour Apparently the only subject who showed a peak blood concentration as much as 2 hours after administration had a rather slow absorption rate This is consistent with the fact that the same subject also showed low plasma concentration when noscapine was administered in divided doses or as the resin complex

From fig 2 it can be seen that an increase in dose resulted in a more than proportional increase in plasma concentration If for example, the dose was increased from 2 to 4 mg/kg, there was a five-fold or greater increase in the plasma concentration From the same figure it can also be seen that clinical doses of noscapine chloride (20–50 mg = 0 3–0 8 mg/kg) should result in plasma concentrations hardly measurable by our method Administration of 4 mg/kg, corresponding to about ten times the clinical dose, gives a concentration of about 0 5 µg/ml after two hours If the drug is evenly distributed in the aqueous phase of the organism, the concentration should be at least ten times as high as that actually found Table 1 shows that after intravenous administration to rabbits only 5% of the noscapine was found in the circulating blood a few minutes after injection and less than 0 5% after 1 hour

The rapid disappearance of noscapine from the blood in human subjects cannot be caused by renal excretion in none of the experiments was the total excretion of noscapine more than about 1% of the dose given during the first 6 hours These results agree completely with those of COOPER & HATCHER (1934) in cats Excretion by rabbits has not been examined

Further, the rapid disappearance of noscapine from the circulating blood cannot be due to a rapid transformation to some metabolite undeterminated by the method used The result of such a rapid transformation would be that after a suitable fractional intake no measurable plasma concentration would be obtained However, as seen in fig 3, and in 5 similar experiments not reported here, this was not so With fractional intake at a fairly slow rate, the plasma concentration first became measurable when the total dose exceeded about 100 mg and from then

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Porphyria Induced in the Rabbit by Diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate

I Excretion of δ-aminolaevulic Acid, Porphobilinogen, Coproporphyrin and Protoporphyrin

By

Birgitta Haeger-Aronsen

(Received April 11 1961)

Two clear cut types of experimental hepatic porphyria have so far been described one is induced by derivatives of α substituted allylacetic acid the other by hexachlorobenzene The "haematoporphyrinuria" observed by STOKVIS (1895) and others in sulphonal-poisoned rabbits may represent a third type, but the methods available at that time did not permit more detailed studies of the pigments excreted

The former type was first observed in rabbits (SCHMID & SCHWARTZ 1952b) receiving toxic doses of allylisopropylacetylcarbamide (sedormid ® = apronal (NFN)) Its occurrence has since been confirmed by other workers (BÉNARD, GAJDOS & GAJDOS TOROK 1953, GOLDBERG 1954 and others) The concentrations of the porphobilinogen and uroporphyrin in the urine are markedly increased but the level of urinary δ aminolaevulic acid is normal (STICH 1958) In the acute intermittent porphyria of genetic origin all three metabolites are excreted in excess of normal SCHMID & SCHWARTZ (1952 a) have shown that the catalase activity of liver cells is decreased in sedormid intoxicated rabbits This is not so in patients with acute intermittent porphyria (GRAY 1950)

Another type of aquired porphyria was discovered in Turkey (CAM 1957 1959 cit SCHMID 1960, CETINGIL & ÖZEN 1960, SCHMID 1960) during an epidemic of a syndrome resembling porphyria cutanea tarda The cause was found to be hexachlorobenzene, which had been added to wheat as a fungicide. The patients excreted abnormally large amounts of uroporphyrin and coproporphyrin in the urine and faeces, but the

proportion is reversed, so that the excretion of noscapine after 6-7 hours is almost entirely in the conjugated form (fig 4) It is not known whether or not conjugated noscapine is present in the plasma If plasma is hydrolysed with hydrochloric acid, as described earlier for urine, the samples gel when an ether extraction is attempted, and no separation of the two phases can be achieved It is noteworthy that the excretion of morphine in urine, relative to the dose, is much higher than that found for noscapine After therapeutic doses of morphine PÆRREGAARD (1957) found that about 40–50% was excreted in the urine, about 1/10 of it as free morphine

Summary.

1) By means of a fluorimetric method previously described the blood concentration of noscapine and its excretion in the urine have been determined after oral administration to human subjects

2) The measured plasma concentrations are small and plasma concentration increases much more than corresponding increases of the doses When the alkaloid is administered intravenously to rabbits it disappears almost completely from the circulating blood within a few minutes

3) Measurements of the blood concentrations at various times after the intake of the alkaloid give a possible index for the absorption rate

4) When noscapine is administered as a resin complex, the blood concentration curve is similar to the curve obtained when it is administered in divided doses as the chloride

Acknowledgment
I am grateful to Miss Hanne Tvilling for valuable technical assistance

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The δ aminolaevulic acid and porphobilinogen in the urine were determined by the methods of MAUZERALL & GRANICK (1956) coproporphyrin was measured by a modification (HAEGER ARONSEN 1960) of ASKEVOLD s method (1951) Determinations

constants and correction divisors of WITH (1955) were used

The concentration of haemoglobin and the numbers of red and white blood cells were determined before the first and after the last dose of DDTD

Glucose and albumin in the urine were measured qualitatively with clinistix ® and albustix ® (Ames analytical reagents) respectively

Results.

The mean values found for the daily excretion of δ aminolaevulic acid, porphobilinogen and coproporphyrin in the urine are given graphically in fig 1

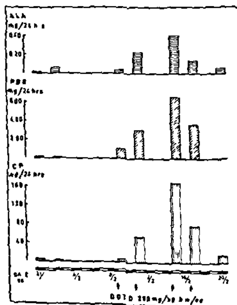


Fig 1 Mean urinary excretion of δ-aminolaevulic acid (ALA) porphob... and copro...
Arrows indicate days of ... trimethylpyr...

It is clear from the figure that the excretion of all three metabolites increased after administration of DDTD The curves for the excretion were similar in shape, and they rose during the period covered by the first three doses, but then began to fall despite administration of the

excretion of porphobilinogen was found to be normal (CETINGIL & ÖZEN 1960) The excretion of δ-aminolaevulic acid was not studied

A corresponding increase in excretion of porphyrins has also been found in rats poisoned with hexachlorobenzene (SCHMID 1960, OCKNER & SCHMID 1961) The animals also excreted an excessive amount of δ-aminolaevulic acid and porphobilinogen in the urine, though not until 2–8 weeks after administration of hexachlorobenzene had been begun The concentrations of uroporphyrin, coproporphyrin and protoporphyrin in the liver were raised, but the increases were not accompanied by any demonstrable decrease in catalase activity of the liver cells

When studying the uptake of orally administered fluorescent substances by the lining of the forestomach in mice, FIGGE (1959) fortuitously observed that in animals receiving diethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate (DDTD), which has a blue fluorescence, the liver and the gall-bladder exhibited a bright red fluorescence in near ultraviolet light SOLOMON & FIGGE (1959) have shown that the concentration of protoporphyrin and coproporphyrin is markedly increased in the liver of mice receiving DDTD They studied the excretion of porphyrin in a DDTD-intoxicated guinea-pig, which had also received the preparation by mouth, and they found the urinary coproporphyrin to be raised Examination by the semi-quantitative method of WATSON & SCHWARTZ (1941) showed only traces of porphobilinogen in the urine They compared the effect of sedormid and DDTD on porphyrin metabolism and found that both substances raised the concentrations of protoporphyrin and coproporphyrin in the liver, but only after administration of sedormid did they find any increase in urinary porphobilinogen Though green porphyrins are abundant in the liver of rats receiving sedormid (SCHWARTZ & IKEDA 1955), SOLOMON & FIGGE (1960) were unable to demonstrate the occurrence of these pigments in DDTD intoxicated mice

This paper is concerned with the excretion of δ-aminolaevulic acid, porphobilinogen, coproporphyrin and protoporphyrin in DDTD fed rabbits

Material and Methods.

Six rabbits (3 males, 3 females) weighing between 2 4 and 2 7 kg were given diethyl [illegible] carboxylate (DDTD) (Eastman Kodak Co, [illegible] weight 4 times during 8 days (see fig 1) [illegible] 6% alcohol and administered by stomach [illegible] Immediately afterwards about 20 ml of water were given by the same route [illegible]

of urine and faeces were collected separately

DDTD is not closely related to sedormid or hexachlorobenzene DDTD, like hexachlorobenzene, increases the urinary δ aminolaevulic acid, but sedormid does not (STICH 1958) Biochemically, then, porphyria induced by DDTD or hexachlorobenzene resembles acute intermittent porphyria more closely than does porphyria induced by sedormid (table 1)

The catalase activity of the liver in DDTD intoxication is the subject of a paper in preparation

Summary.

Oral administration of diethyl 1,4-dihydro 2,4 6-trimethylpyridine 3,5-dicarboxylate (DDTD) to rabbits produces porphyria, characterized by a markedly increased excretion of porphobilinogen and coproporphyrin and a moderately increased excretion of δ aminolaevulic acid in the urine

The faecal protoporphyrin and coproporphyrin are raised, particularly the former

Acknowledgement

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fourth dose The excretion of *porphobilinogen and coproporphyrin* was much larger than that of δ-aminolaevulic acid Thus, after the administration of the third dose, the mean excretions of porphobilinogen and of *coproporphyrin had increased forty fold*, but that of δ aminolaevulic acid was only ten times the original value

The increased excretion of porphyrin and porphyrinprecursors in the urine was accompanied by a marked rise in the excretion of porphyrins in the faeces The increase in faecal porphyrins was due mainly to a rise in protoporphyrin

No change was noted in excretion of the metabolites by the control animals

The intoxication was not accompanied by any significant change in the concentration of haemoglobin or in the numbers of red or white blood cells

Neither glucosuria nor albuminuria was demonstrable

Towards the end of the experimental period the animals appeared weak, and they had no appetite No signs of paralysis were observed

Comment.

The investigation showed that DDTD produces a prompt and considerable rise in the urinary porphobilinogen, coproporphyrin and δ-aminolaevulic acid in the rabbit

The results differ from the findings of SOLOMON & FIGGE (1959, 1960) in so far as these authors, who used the semi quantitative method of WATSON & SCHWARTZ (1941) for determination of porphobilinogen, did not find any increased excretion of the metabolite in DDTD intoxicated rats or guinea-pigs

Table 1

Urinary δ aminolaevulic acid porphobilinogen and coproporphyrin and faecal coproporphyrin and protoporphyrin in patients with acute intermittent porphyria and in animals with experimental porphyria

Type of porphyria	Urine			Faeces	
	δ amino laevulic acid	porpho bilinogen	copro porphyrin	copro porphyrin	proto porphyrin
Acute intermittent porphyria (human beings)	++	+++	(+)	+	+
Sedormid induced (rabbits rats)	–	+++	+	+	++
Hexachlorobenzene induced (rats)	++	+++	+	+	+
DDTD induced (rabbits)	+	+++	+++	+	++

tion and once on each subsequent day Only the pseudocholinesterase activity was determined and the result given in µmols of acetylcholine split per min/ml serum (37°C and pH 7 4) By this method the normal range for men is found to be 3 20–5 68 µmols per min /ml (mean value ± 2 x the standard deviation) and for women 2 28–5 00 µmols per min /ml

Small doses of DFP inactivate the pseudocholinesterase in the serum, but exert no notable effect upon tissues and little effect upon the specific erythrocyte cholinesterase (HAWKINS *et al* 1947) However, the tissues show increased sensitivity to acetylcholine, but as a rule without exhibiting cholinergic signs

Table I

Acetylcholinesterase activity after i m injection of 0 6 mg DFP, measured in µmols ACH/min /ml plasma

Patients	Case	Before	Days after injection of di isopropyl fluorophosphate						
			1	2	3	4	5	6	8
Men	1	4 62	2 10		2 64	2 60		2 86	2 96
	2	4 50	1 95		2 84	1 82		3 64	3 20
	3	4 14	2 13		3 10			3 46	4 10
	4	5 38	2 60		3 36	3 44		3 60	3 76
	5	3 86	2 12			2 76		3 64	
	6	2 76	1 55		1 70	1 76		1 96	
	7	5 36	1 90		3 60	5 02		4 07	
	8	5 16			3 30	3 06		3 76	3 44
Women	9	5 44	2 12		2 82	2 84		3 02	3 16
	10	5 96	4 10		3 36	3 82		3 80	3 40
	11	6 40	2 80	3 24		3 20		3 62	4 24
	12	4 06	1 55		2 10			2 60	2 70
	13	5 64			2 76		3 74		3 30

Results.

The findings are given in table I and in fig 1 Twenty four hours after the injection the pseudocholinesterase activity was distinctly reduced, on the average to less than half the initial value JANDORF *et al* (1950) found a maximum reduction in esterase – both in the plasma and in the red cells – shortly after intravenous injection into rabbits, 24 hours after the injection the concentration had again increased considerably Probably, therefore, the initial fall in our series also was considerably greater than the fall measured the first determination was not carried out until 24 hours after the injection In one subject the minimum value was found on the 2nd–4th day after the injection MARCHAND (1952) found remission in pseudocholinesterase to about one half within 5 days, and normal values were re-established in 3 weeks (cf KARCZMAR *et al* 1949)

At no time did our patients show any symptoms or signs that could have been attributed to a toxic effect of the DFP

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Cholinesterase Activity of Human Plasma after Intramuscular Diisopropyl Fluorophosphonate (DFP)

By

Troels Munkner, Jorgen Matzke, and Aage Videbæk

(Received April 18 1961)

The use of DFP for determining the life span of the red blood cells was suggested by GROB *et al* (1947), and DFP^{32} was introduced for this determination in human subjects as well as in animals by COHEN *et al* (1954) In cross-transfusion studies MUNKNER *et al* (1960) used Cr^{51} labelled red cells and red cells labelled with DFP^{32} Labelling with DFP in assessing the life span of the cells involves the advantages, among others that it can be carried out *in vivo* and that the labelling of the individual erythrocytes is apparently stable until haemolysis sets in

Before using DFP, however, it must be realized that it alters cholinesterase activity Alkyl- and aryl fluorophosphonates are anti cholinesterases, which bind themselves irreversibly to cholinesterases, including the specific cholinesterase of the red cells and the non-specific pseudo cholinesterase of the plasma (MENDEL *et al* 1943, HAWKINS *et al* 1947)

A study of the decrease in cholinesterase activity after injection of DFP, combined with a clinical assessment of the patients' condition, may help in discussing of the value of determining esterase activity after poisoning with insecticides, such as parathion, or with nerve gases The clinical manifestations of poisoning due to inhibition of cholinesterase after the ingestion of toxic quantities are miosis, blurred vision, headache and fatigue, increased secretion of sweat and saliva, vomiting and diarrhoea, dysphagia respiratory failure, muscular fibrillation and convulsions Death is caused by respiratory paralysis

Material

[illegible] males and five females suffering from various diseases were injected intra

Discussion.

HAWKINS *et al* (1947) claim that determinations of pseudocholinesterase do not give a true picture of the correlation between anticholinesterase effect and pharmacological effect, since it is the specific cholinesterase that is responsible for the hydrolysis of acetylcholine in the nerve endings, not the non specific serum esterase FRAWLEY *et al* (1952), hold that determinations of pseudocholinesterase are of value in confirming exposure, but less so in assessing the degree of the exposure or the severity of any symptoms They state that much the same reduction in plasma (and erythrocyte) cholinesterase activity may be found after single doses ranging from those producing no symptoms to the lethal SUMERFORD *et al* (1953) reported mild clinical symptoms and signs when there was a slight reduction in activity to below the average normal range, whereas severe poisoning was seen only along with a marked reduction in true cholinesterase ANDERSEN *et al* (1952) have described a case of poisoning with clinical symptoms in which the activity fell only to about 60% of the control value KARLOG *et al* (1958) have observed clinical well being while the cholinesterase activity was only a few per cent of normal in blood cells as well as in plasma ATHENS *et al* (1959) used DFP on 120 persons who only had local pain after the intramuscular injection, but no systemic symptoms or signs on doses of up to 3 mg DFP A dose of 4 mg gave rise to symptoms in 3 out of 4 subjects

In our series the activity fell, as already mentioned, to an average of 50% of the initial value without any toxic signs

Conclusion Our results are in keeping with those reported previously In assessing the degree of acute poisoning, determination of pseudocholinesterase is of little value, though a fall in pseudocholinesterase activity is a valuable indicator of exposure to DFP The use of a small quantity of DFP for labelling erythrocytes must be considered permissible, as none of our patients developed toxic symptoms or signs

Summary.

Di isopropyl fluorophosphonate in doses of 0 6 mg i m was administered to 8 men and 5 women The effect of DFP upon the pseudocholinesterase activity in plasma was measured This revealed a distinct fall in activity, but clinical signs of intoxication did not appear in any subject This confirms that alterations in the pseudocholinesterase activity are of limited value for assessing the toxic effect of DFP The half-life of the pseudocholinesterase was found to be 16 days (cf fig 2)

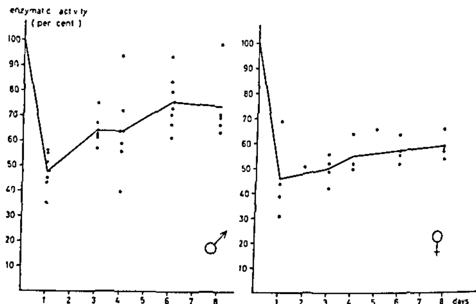


Fig 1 Alterations in pseudoacetylcholinesterase activity in the plasma of 8 men and 5 women observed for up to 8 days after i m injection of 0 6 mg DFP The solid line gives the average values the dots indicate the individual values

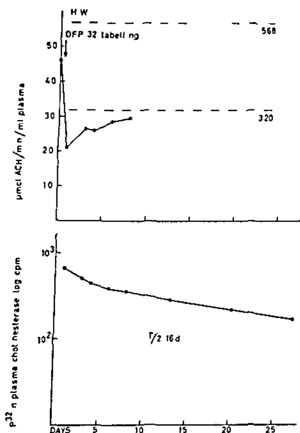


Fig 2 *Upper curve* Cholinesterase activity before and after i m injection of DFP, 0 6 mg, into a normal individual *Lower curve* Concentration of DFP[32] labelled plasma cholinesterase plotted against time (same patient as for the upper curve)

Acta pharmacol et toxicol 1961, 18, 175–178

From the Medical Department B Copenhagen County Hospital, Glostrup
(Head Erik D Bartels)

The Effect of Probenecid on Renal Excretion and Plasma Level of Uric Acid in Patients with Renal Insufficiency

By

N R Haagensen and Bent Nielsen

(Received April 12 1961)

Renal excretion of uric acid is, both in normal individuals and in patients with gouty arthritis, greatly increased after oral administration of probenecid, and the uric acid clearance rises considerably Initially the serum uric acid level falls Administration of 1–4 g probenecid daily for several days however, induce a rise in uric acid production in half of the persons investigated (CRONE & LASSEN 1955, GJØRUP & POULSEN 1955)

In patients suffering from chronic renal insufficiency an elevated serum uric acid concentration is often encountered, and some of these patients in fact experience gouty attacks

As different subfunctions of the kidneys may be disturbed, probenecid might not be expected to be able to increase the urinary excretion of uric acid in all of these patients If no uricosuric effect is obtained, an eventual peripheral effect upon production or mobilization of uric acid could bring about a rise in the serum concentration of uric acid, not desirable in these patients Whether this is indeed so has now been investigated

The results indicate that probenecid can be administered to patients with renal insufficiency with no danger and without any further rise in the serum concentration of uric acid

Material and Methods.

The patients These were 9 females from 32 to 72 years of age admitted to the Department for renal insufficiency

Diagnosis 7 patients had chronic pyelonephritis of a few months to 20 years duration in most cases from 1 to 5 years The duration of the disease in several patients was, however somewhat uncertain

One patient (no 5) had a chronic nephritis first recognized during pregnancy 18 years earlier She had hypertension (200 130 mm Hg) and hypertensive heart disease

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Results.

Six of the eleven patients tested showed before the investigation serum concentrations of uric acid above normal (table 1) No correlation between either the serum creatinine concentration or the haemoglobin value and the serum uric acid level could be found Three patients showed a fall in the serum uric acid concentration, the rest showed no significant change in either direction

The mean values before and after probenecid administration were 76 and 68 mg/l, respectively (fig 1)

Seven patients showed a rise in urinary excretion of uric acid, two showed a decrease, in one patient the excretion was not altered after administering probenecid No correlation could be found between degree of renal insufficiency and effect of probenecid The mean values before and after probenecid administration were 325 and 568 mg/24 h, respectively (fig 2)

It has been demonstrated that probenecid can increase the urinary excretion of uric acid even in patients with severe renal insufficiency No simultaneous rise in serum uric acid concentration was found, and no other untoward reactions were observed It thus seems safe to treat uraemic patients with probenecid if nessesary

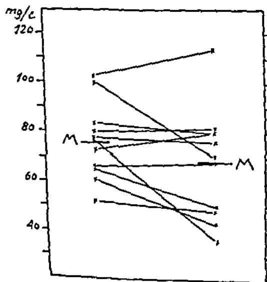


Fig 1 Serum concentration of uric acid (mg/liter) before and after ingestion of 1 g of probenecid and 5 g of sodium bicarbonate M = mean value

with congestive failure In 1 patient (no 3) no specific diagnosis has been established but she had a pronounced renal insufficiency

Complications Of the seven patients with pyelonephritis, 2 had moderate hyper tension (no 7 Max 195/110, no 8 Max 170/120 mm Hg) Papillary necrosis verified by typical urograms or by passage of renal papillae was encountered in 5 patients with pyelonephritis, one of these had renal calculi Diabetes mellitus was not found in these subjects

Clinical symptoms Symptoms of uraemia (nausea, vomiting thirst anaemia con vulsions, somnolence, haemorrhagic diathesis) were found in all but one patient (no 2), the most common symptom being anaemia

All of the patients had impaired concentration capacity, as measured by the maximal spontaneous specific gravity of the urine

Cardiac failure was found in 2 patients with pyelonephritis (no 1 and 8) and in patient no 5, as mentioned above In 6 of 7 cases of pyelonephritis excessive intakes of phenacetine-containing analgaesics have been recorded in most this abuse had lasted for several years

Two patients with chronic nephritis and undefined kidney disease had not taken undue amounts of analgaesics

None of the patients experienced gouty attacks

Experimental Procedure

In patients not confined to bed were investigated A liberal diet was allowed

For 5 days daily determinations of uric acid concentrations in serum and urine were performed Samples of urine from every 24 hour period, beginning at 9 p m, were used A small amount of chloroform was put into the vessel containing the 24 hour urine Venous blood samples were drawn at 8 a m On the third day an oral dose of 1 g probenecid and 5 g of sodium bicarbonate were given at 9 p m

To compare serum concentrations, the mean values for two days before and two days after administering probenecid were used To compare urinary excretions the mean value of the total excretion for two days before administering probenecid and the highest value for the two days after were used The uric acid was determined in the Hospital Central Laboratory by the enzymatic method described by PRÆTORIUS (1949)

Table 1

Patient no	Age	Hgb g/l	Serum creatinine mg/l	Standard bicarbonate m Eq/l	Uric acid			
					Serum concentration mg/l		Excretion in urine mg/24 h	
					before	after	before	after
1	38	93	89	21	61	43	280	337
1a	39	75	138	18	77	36	265	1164
2	32	133	18	20	52	48	349	778
3	70	104	64	20	103	114	210	264
4	73	102	26	22	65	50	372	248
5	54	120	50	22	77	77	425	383
6	55	75	40	17	101	71	316	378
7	52	107	66	15	66	68	360	385
8	38	116	110	20	80	92	394	1068
9	62	99	50	21	73	81	303	676
9a	62	103	61	18	84	81	297	-

Acta pharmacol et toxicol 1961, 18, 179–182

From the Department of Pharmacology and Toxicology, Royal Veterinary and Agricultural College, Copenhagen (Professor Sv Dalgaard Mikkelsen V M D)

Toxic Effect of Monochloracetate on Geese

By

M Christiansen and Sv Dalgaard Mikkelsen

(Received April 19 1961)

To facilitate the work of harvesting unequally ripened crops of clover, and to reduce the risk of loss, farmers use, among other things, the sodium salt of monochloracetic acid as an agent to produce early ripening A few days after spraying with 20 kg per hectare dissolved in 500 l of water the crop will have withered and dried up, being then ready to be harvested mechanically Since monochloracetate is used on seed-crops and is decomposed by soil bacteria (JENSEN 1957, 1959, 1960), the chance of its poisoning domestic animals is regarded as negligible The same has been claimed to apply to game but in 1958 a report was received of poisoning after spraying that killed a flock of wild geese (Grey Lag Goose, *Anser Anser L)*

ROSENORN-LEHN (1958–1959) reports as follows

On August 4 1958 between 10 a m and 4 p m , a white clover field of 18 hectares was sprayed with a solution of 18 kg sodium monochloracetate in 400 l of water, to which had been added a 1% (v/v) solution of sulphonated detergent per hectare About [illegible] clover f[illegible] This cr[illegible] field wa[illegible] The geese therefore sought their food in the newly sprayed clover field The next few days the geese were not observed, and they were then all found dead on their reservation

Everything pointed to poisoning with monochloracetate As, however, the geese were in a state of advanced decay and disintegration, this diagnosis could not be confirmed We therefore thought it appropriate to investigate the toxic effect of monochloracetate on geese

Material and Methods.

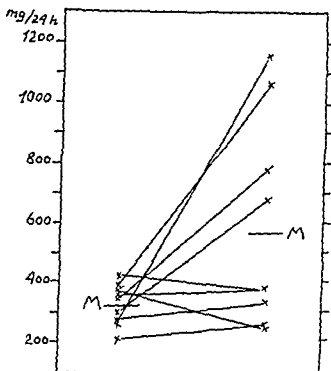


Fig 2 Urinary excretion of uric acid (mg/24 h) before and after ingestion of 1 g of probenecid and 5 g of sodium bicarbonate M = mean value

In these 'acute' experiments no increased production of uric acid has been demonstrated after administering probenecid The results from three patients showing a considerable rise in urinary excretion with no fall, or even a slight rise in serum concentration of uric acid is not in consistent with such an effect of probenecid Several patients, however, showed no rise in either urinary excretion or serum concentration of uric acid

Summary.

Patients with chronic renal insufficiency were investigated for the effect of probenecid on their urinary excretion and serum concentration of uric acid Probenecid increased excretion in the urine without any major alteration in serum concentration

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membrane was seen to be diffusely reddened and spotted with punctate haemorrhages The tracheal mucosa showed vascular congestion

The chemical analyses of organ material gave the results set out in table 1 It is seen that only the geese given sodium monochloracetate by tube and submitted to post mortem examination shortly after death had larger amounts of organically bound chlorine in the gizzard and intestinal contents than the controls The concentrations in homogenised liver tissue were always found to be of the same order as that in the control liver from an untreated goose of the same flock

Discussion.

The investigations reported here show the lethal dose of monochloracetate for geese to be identical with that reported by WOODARD, LANGE, NELSON & CALVERY (1941) for rats and guinea pigs (LD50 about 75 mg/kg) As farmers spray sodium monochloracetate for early ripening of clover at a concentration of 2000 mg per square metre, there is, as shown, a great risk of fatal poisoning of geese foraging on the crop Walking amongst a freshly sprayed plot without feeding, on the other hand, causes no poisoning This is in agreement with the practical observation that among such wild birds as pheasants and partridges, which mainly seek animal food in clover fields, the mortality has not been found abnormally high during the period of spraying for early ripening of clover

The picture of poisoning in geese has features in common with that of monobromacetic acid poisoning in pigs and dogs as described by DALGAARD-MIKKELSEN, KVORNING & MØLLER (1955) and ANDERSEN, DALGAARD-MIKKELSEN & KVORNING (1955) They are both characterised by an effect on muscular activity and by a latency of some hours from intake to a sudden development of clinical signs For wild geese this latency means that they can move far from their feeding place before the poison begins to interfere with their mobility

The fact that an increased content of organically bound chlorine was only demonstrable in perfectly fresh post mortem material suggests that monochloracetate, like other organic chlorine compounds (BRAY, THORPE & VALLANCE 1952) and monobromacetic acid (HANSEN 1956), is decomposed rapidly in a biological environment Consequently a toxicochemical analysis does not always serve as a reliable guide in practice

Summary.

A case is reported of monochloracetate poisoning of wild geese after they had fora[illegible]
chloracetate [illegible]

allowing them to forage on a clover plot 1 hour after an aqueous solution had been sprayed or by administering through an oesophageal tube an aqueous solution with added agar

Chemical determinations of organically bound chlorine in the contents of gizzard and intestines and in the liver were performed by HANSEN s modification (1956) of the CONWAY method (1950)

Results

Six geese were transferred to a clover plot, where they had never been before, 1 hour after this had been sprayed with the quantity of sodium monochloracetate solution used in normal practice, corresponding to 20 kg per hectare in 500 l of water The geese moved about in the moist clover, but ate practically nothing No signs of poisoning were observed over the next 48 hours

Spraying over areas where the geese felt at home, and therefore ate freely, led to the deaths of four out of six geese within 24 hours of spraying with 20 kg sodium monochloracetate per hectare In another experiment with 40 kg per hectare three out of six geese died

Administration by oesophageal tube of 50 mg per kilogram body weight gave no signs in two geese Administration of another 75 mg/kg one week later killed both geese after 4 and 6 hours A dose of 100 mg/kg to two geese resulted in death within 3½ hours It was characteristic, both after intake with fodder and after administration by oesophageal tube that the geese were apparently unaffected until about 3 hours afterwards Then smacking sounds and acts of deglutition were noticed, as well as unsteady gait, during which the test birds almost literally lost their heads, the neck falling backwards on to the back The geese developed intermittent convulsions 15 to 30 minutes before the occurrence of death in reflexless coma, in between they lay with extended wings and legs, attempting feebly to raise the head by the neck

At necropsy all the test birds were found with thin and slimy contents of oesophagus and glandular stomach In the duodenum the mucous

Table 1

Organically bound chlorine in gizzard and intestines and in liver µg/g

	Control	Residue left on clover							Administered by oesophageal tube			
		20 kg/ha				40 kg/ha			75 mg/kg		100 mg/kg	
Goose no	0	1	2	3	4	7	8	9	a	b	c	d
Intestines	11	24	16	27	17	15	17	25	47	34	205	106
Liver	35	39	27	23	19	24	25	33	40	23	42	39

Acta pharmacol et toxicol 1961, 18, 183–190

Department of Pharmacology, Royal Veterinary College Stockholm, Sweden

Biological Fate of C^{14}-labelled Ethyl (3,3-diphenyl-1-methyl-propyl) dimethylammoniumbromide Cetiprin®, a Monoquaternary Ammonium Compound

By

Eskil Hansson and Carl G Schmiterlow

(Received April 30 1961)

The physiological disposal of a number of monoquaternary ammonium compounds has been reported by LEVINE & CLARK 1955, 1957 and LE VINE, BLAIR & CLARK 1955 Their results show that the pattern of tissue distribution is approximately the same for all these substances despite structural variations The quaternary ammonium compounds examined were found to be distributed primarily in the tissues concerned with excretion, but the concentration of the drugs in other organs was too low to be determined by the chemical method employed In order to investigate [illegible]

[illegible] drug has been studied in mice and rats by autoradiography and radioassay

Methods.

Synthesis of C^{14} Cetiprin[2])

The synthesis of C^{14}-cetiprin was carried out as described below

1-C^{14}-ethyl(3 3-diphenyl 1 methylpropyl) dimethylammoniumiodide was prepared by reacting C^{14} 1-ethyliodide (The Radiochemical Centre [illegible]

4 4-diphenyl 2-dimethylamino-n butane [illegible]

[illegible]

[illegible] spot was found on the chromatograms.

[1]) Recip Co Stockholm Sweden

[2]) We are indebted to Mr S Carlsson and Mr N Forsberg Recip Co for synthesis of the labelled Cetiprin

For domestic geese the lethal dose of sodium monochloracetate after oral intake or administration has been found to be of the order of 75-100 mg/kg.

Acknowledgement.

The expenses of our investigations were defrayed by the Danish Game Foundation. The State Veterinary Serum Laboratory kindly placed various out-door areas at our disposal, and the State Department of Weed Research conducted the spraying with sodium monochloracetate solutions.

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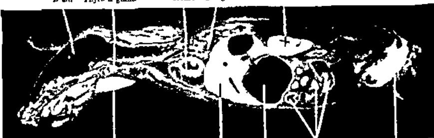


Fig 1 Auto-radiogram showing distribution of radioactivity (light areas) in a mouse 1 hour after intramuscular injection of C^{14} labelled cetiprin

unlike what was found in the blood vessels, was observed in the brain or in the spinal cord

Initially up to one hour, the liver showed the highest concentration of radioactivity, but this level decreased rapidly, indicating secretion via the bile A high activity was seen in the biliary ducts, the gall bladder and the small intestines The accumulation of radioactivity in the intestines is probably mainly due to excretion via the bile (table 2)

The gastric mucosa showed a higher level of radioactivity in its fundic and pyloric parts than that in the intestinal mucosa (fig 3)

The salivary glands showed a high accumulation of radioactivity, but much lower than that in liver and intestine Accumulation of radioactivity was observed in the thyroid gland and radioactivity was found there during the whole period of observation

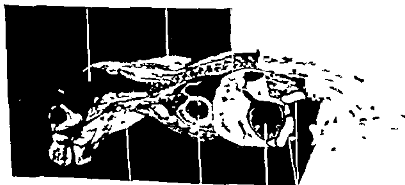


Fig 2 Auto-rad ogram showing d str bution of rad oactivity (light areas) in a mouse 8 hours after intramuscular inject ons of C^{14} labelled cetiprin

Autoradiography

White mice weighing about 20 g were given C^{14}-cetiprin either by stomach tube or by intramuscular injection The dose was 50 µg per gram of body weight (0 16 µC per gram) The mice were killed *15, 30 minutes, 1, 4 8, 24 and 48 hours after dosing* by immersion in a mixture of acetone and solid carbon dioxide (−80°) The frozen mice were mounted in a cold room (−10°) on large stages fitted to a sledge microtome Sagittal sections through the whole animal were taken and dehydrated at −10° Autoradiography was performed by the method of ULLBERG (1954) Exposure was made against an INDUSTREX (KODAK) X ray film or STRUCTURIX (GEVAERT) X ray film Pregnant mice were also used to study passage of the drugs from mother to foetus

Excretion Studies

1 *Urine and faeces* Adult male rats weighing about 300 g were used The animals received the substances intramuscularly or by stomach tube they were kept in metabolism cages allowing the separate collection of urine and faeces and were fed *ad libitum* on a standardized diet

2 *Urine and bile* Adult male rats *weighing about 300 g were used* The rats were anaesthetized with pentobarbital, a polythene tube was introduced into the common bile duct, and the bile was collected The urethra was ligated, and the drugs were injected intravenously Four hours after injection the rats were killed, and the urine was collected from the bladder

Radioactive Assay

Urine, faeces and bile were plated on aluminum planchettes and counted in a gas-flow counter (Tracerlab Inc U S A) Appropriate self absorption corrections were applied when necessary

Metabolism Studies

The radioactive constituents of the urine and the bile were separated by paper chromatography (Whatman No 1) Auto radiograms of the paper strips were prepared on No Screen X ray film Two solvent systems were used The developing solvents *were I) a mixture of n butanol acetic acid water (4 1 5), II)* N butanol and 2 N ammonium hydroxide (5 2)

The chromatograms were developed by descending chromatography with solvent I and ascending chromatography with solvent II

Results.

Auto-radiographic Studies

Examples of the distribution of C^{14}-cetiprin at various times after intramuscular injection are given in fig 1–3 The level of radioactivity in blood was found to be low during the entire period of observation, in spite of the continuous absorption from the site of injection The radioactivity was thus rapidly cleared from the blood with accumulation in the liver, the intestines, the gastric mucosa and the kidneys The lung, fat tissue and connective tissue showed low concentrations No radioactivity,

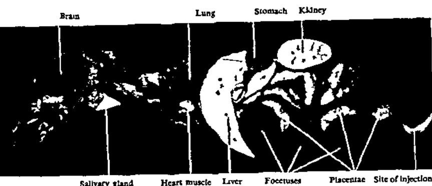


Fig 4 Auto-radiogram showing distribution of radioactivity (light areas) in a pregnant mouse with foetuses in situ 1 hour after intramuscular injection of C^{14} labelled cetiprin Note absence of radioactivity from the foetuses and the brain

The radioactivity was initially low in the skeletal muscles, but after 24 hours the relative concentration had increased considerably The heart muscle showed an especially high concentration

Although the placenta contained a large amount of radioactivity, little or no radioactivity was seen in the foetuses (fig 4)

Autoradiography after oral administration showed that C^{14}-cetiprin was absorbed slowly from the gastrointestinal tract Most of the radioactivity was in the stomach and the intestines one hour after the injection Radioactivity was, however, observed in the liver, the kidneys and the salivary glands Auto-radiograms taken 4 and 8 hours after the injection showed a considerable amount absorbed and approximately the same tissue distribution as efter intramuscular injection

Excretion Studies

The daily output of cetiprin or its metabolites or both in urine and faeces after oral administration and intramuscular is given in table 1

After oral administration most of the radioactivity was eliminated in the faeces during the first day, and the urinary excretion did not exceed 16 1% of the dose administered

After intramuscular administration, the radioactive material excreted in the faeces approximated to that in the urine Between 66 4 and 80 6% of the dose administered was recovered during the three day experiment However, after oral administration 90 5–98 8% was recovered The comparatively low radioactivity recovered after intramuscular injection was probably due to slow absorption of the substance from the site of injection



Fig 3 Enlargement from fig 2 showing accumulation of radioactivity (light areas) in the gastric mucosa 8 hours after intramuscular injection of C^{14} labelled cetiprin

Table 1.

Excretion of C^{14}-cetiprin in urine and faeces

Rat No	Route of administration	Per cent of dose excreted								
		1 d		2 d		3 d		Total		Grand total
		urine	faeces	urine	faeces	urine	faeces	urine	faeces	
IV	per os	4 6	78 3	2 3	8 9	0 9	3 6	7 8	89 8	97 6
V	per os	6 3	90 1	2 9	8 9	0 2	4 4	9 4	85 4	92 8
VI	per os	13 7	59 8	1 5	12 8	0 9	2 6	16 1	74 9	90 5
VII	intramuscular	28 8	34 4	10 8	3 3	0 8	2 5	40 4	40 2	80 6
VIII	intramuscular	28 7	9 3	3 5	20 1	0 8	4 0	33 0	33 4	66 4

Table 2.

Excretion of C^{14}-cetiprin in urine and bile

Rat No	% of total dose collected in urine	% of total dose collected in bile
I	9 2	13 1
II	13 8	18 8
III	10 2	15 1

that three radioactive substances were present (fig 5) The main portion corresponded to unchanged cetiprin The additional weak spots suggest metabolites or conjugation products at low concentrations Tissue extracts from liver, lung and heart muscle contained only unchanged cetiprin

Discussion

The metabolism studies showed that C^{14}-cetiprin was mainly eliminated unchanged, and only traces of metabolic or conjugation products appeared on the chromatograms The chromatograms of liver, lung and heart muscle extract showed only unchanged cetiprin These findings allow us to conclude that the radioactivity present from the tissues on the autoradiograms was mainly unchanged cetiprin

The autoradiographic studies showed a high concentration of cetiprin in organs mainly concerned with excretion of the drug viz liver, intestine, kidney and probably salivary glands In addition there was a marked accumulation in the gastric mucosa where it remained for a comparatively long time without the appearance of any appreciable amounts in the lumen This is of special interest for clinical application of the drug to peptic ulcer

Cetiprin a quaternary compound, did not pass the blood brain barrier The inability of such compounds to pass into the brain has been attributed to their low lipid solubility (BRODIE & HOGBEN 1957)

A high accumulation was seen in the thyroid gland Radioactivity could also be seen in the heart and other muscles These findings are contrary to those of LEVINE, BLAIR & CLARK (1955) but agree with the findings of *ALLGEN et al* 1960 and HANSSON & SCHMITERLOW (1961) for quaternary phenothiazine compounds The accumulation of quaternary compounds in the thyroid and heart muscle is difficult to interpret No toxic reactions on the thyroid or heart have been reported, and no pharmacological effects on these organs have been observed

Quaternary ammonium compounds are known to be absorbed slowly from the gastrointestinal tract (*LEVINE, BLAIR & CLARK 1955*) On oral administration of C^{14}-cetiprin to rats, 4 5–16 1% was recovered in the urine These figures correspond to amounts of drug absorbed from the gastrointestinal tract Since a part of the drug absorbed is eliminated by biliary secretion into the intestine, the amount absorbed is probably much higher than indicated by the recovery in the urine

Summary.

Th

cet

Since large amounts of radioactivity were seen in the small intestines, experiments were conducted to determine the manner in which the compound reached the intestine Samples of bile were collected and analysed for radioactivity Output of radioactivity in the bile and in the urine was determined during a four hour period after intravenous injection of C^{14}-cetiprin (table 2) The results indicate that after intravenous injection

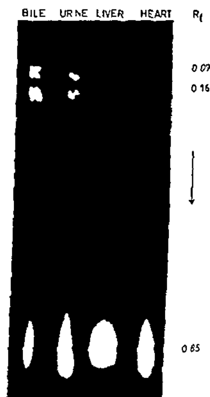


Fig 5 Auto radiogram of paper chromatogram showing radioactivity (light areas) corresponding to unchanged cetiprin (R_f 0 85) and conjugates (R_f 0 09 and R_f 0 16) The chromatogram was developed by descending chromatography with n butanol acetic acid water (4 1 5) as solvent For further explanation see text

in the rat a significant amount of cetiprin was excreted via the bile and the urine The percentage of recovery in the bile was found to account for between 13 1–18 8% and in the urine for 9 2–13 8% of the injected dose

Metabolism Studies

These studies were performed on mice and rats Injections were made intramuscularly or intravenously Small measured portions of urine and bile and water extracts from liver, lung and heart muscle were chromatographed on paper Cetiprin is mainly eliminated unchanged The paper chromatography of urine and bile after injection of C^{14}-cetiprin revealed

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The Effect of Intra-arterially Administrated Hydralazine on Blood Flow in the Forearm and Hand[1])

By

Åblad, B, G. Johnsson and M. Henning

(Received May 4 1961)

The hypotensive effect of hydralazine (1 hydrazinophthalazine) in man and animals is due to a reduction in peripheral vascular resistance (MOYER *et al* 1951, WILKINSON *et al* 1952, FREIS *et al* 1953) Animal studies have given rise to divergent opinions whether the site of action is central or peripheral, but there is evidence that after intravenous administration of therapeutic doses of hydralazine in humans the vasodilator effect arises at least in great part from a peripheral point of interference (STUNKARD *et al* 1954, ÅBLAD 1959) WILKINSON *et al* (1952) found that hydralazine injected into the femoral artery, in doses exceeding 20 mg, raised the skin temperature of the ipsilateral but not of the contralateral leg The reported dosage was so high, however, that the hydralazine concentration in the leg may possibly have exceeded the therapeutic level by a substantial margin

Since it seemed of interest to ascertain whether intra arterial administration of lower doses would also give rise to local vasodilation, we infused hydralazine at low dosage into a brachial artery and studied its effects on the blood flow in the forearm [illegible] lood flow was measured by [illegible] & VAN ZWALUVENBERG 190[illegible]

Methods.

The experiments were performed on healthy students in a recumbent position

[illegible]

[1]) [illegible] was presented at the Joint meeting of the British and Scandinavian Pharmacological Societies in København Denmark, July 1960

permitted an autoradiographic study of the distribution and also made it possible to measure and follow its excretion and metabolism

A rapid accumulation of cetiprin was observed at early intervals in the liver, the intestines, the salivary glands and the kidneys The concentration in gastric and intestinal mucosa was high. An accumulation of cetiprin was also observed in the thyroid and muscles. Cetiprin was excreted via the urine and faeces The main route of elimination was in the faeces after oral administration, owing to its low absorption from the intestine. However, after intramuscular injection the excretion in urine and faeces was approximately equal. Cetiprin was also excreted via the bile. The radioactivity in bile, urine and in tissues was shown by paper chromatography to be mainly due to unchanged cetiprin. Traces of metabolites or conjugation products or both in urine and bile were also demonstrated.

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Table 1

Effect of intra arterially infused hydralazine on blood flow in the upper part of the forearm. The drug was infused into the brachial artery of the test arm for five minutes. Blood flow in ml per 100 ml tissue per minute

Exp No	Dose of hydralazine mg	Forearm volume ml		Before infusion		After beginning infusion of hydralazine									
						1–6 min		7–12 min		13–18 min		19–24 min		25–30 min	
		Test	Control	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm
1	0.36	680	660	3.3	3.4	5.4	3.2	10.8	3.7	11.1	3.2	10.5	3.0	10.0	2.6
2	0.24	660	670	6.7	5.8	9.4	7.0	10.8	5.9	11.1	5.7	11.3	5.8	12.2	7.6
3	0.24	590	550	4.2	3.9	5.0	3.7	7.6	4.9	5.8	3.3	5.6	3.1	6.3	3.6
4	0.18	665	650	2.9	2.7	4.0	3.7	4.3	2.5	4.8	2.7	4.7	2.6	5.5	2.4
5	0.18	820	760	7.2	5.2	7.7	4.9	9.7	4.9	10.5	4.7	10.2	4.7	9.7	4.2
6	0.18	680	695	3.1	3.2	3.8	3.4	5.6	3.6	6.1	3.3	5.5	3.2	5.7	3.4
Mean values	0.23	683	664	4.57	4.03	5.88	4.32	8.13	4.25	8.23	3.82	7.97	3.73	8.23	3.97
Mean change in test arm after hydralazine						1.31 ±0.36[1]		3.56 ±0.87[2]		3.66 ±0.92[1]		3.40 ±0.89[1]		3.66 ±0.79[2]	
Mean change in control arm after hydralazine							0.29 ±0.27		0.22 ±0.61		−0.21 ±0.10		−0.30 ±0.13		−0.06 ±0.41
Mean difference between test arm and controlarm				0.54 ±1.26		1.56 ±0.43[1]		3.88 ±0.85[2]		4.41 ±0.94[2]		4.24 ±0.91[2]		4.26 ±0.80[2]	

[1] $P < 0.05$
[2] $P < 0.01$

outer diameter of 0 6 mm was then introduced into the artery, the blood flow to the arm being temporarily occluded by a cuff placed round the arm near the axilla The catheter was advanced proximally for about 8 cm into the artery The catheter was connected to a syringe placed in an infusion apparatus, and through it isotonic sal ne or hydralazine was infused into the artery at a constant rate, usually 0 3 ml per minute

Plethysmographs were then applied to both hands or to the upper part of both forearms and placed at the level of the sternal angle The plethysmographs were of the type described by FOLKOW *et al* (1958) The hands were covered with loose fitting latex gloves and the forearms with sleeves of thin latex

For the determinations of forearm blood flow, the temperature of the plethys mograph water was 34°C and the room temperature 24° ± 1° Cuffs were applied round the arms just proximal and distal to the plethysmographs The blood flow was measured simultaneously in both forearms by rapidly inflating the proximal cuffs about once per minute to a pressure of 50 mm Hg This was done by connecting the cuffs to a constant pressure reservoir of the type designed by HESS (1954) The duration of each inflation was about 15 seconds The volume increase in the forearms was registered on smoked paper by float recorders filled with paraffin The distal cuffs were inflated to 200 mm Hg at least one minute before measurement of the blood flow The hand circulation was blocked during the administration of hydralazine

During measurement of the hand blood flow the temperature of the plethysmo graph water was 33° and the room temperature 27° ± 1° Cuffs were applied just proximal to the plethysmographs and the blood flow was measured in the same way as in the forearm experiments

It will be evident from the results that the blood-flow was approximately equal in the two arms before administration of hydralazine, and there was no indication that the catheterization of one brachial artery interfered with the blood-flow to the hand or forearm

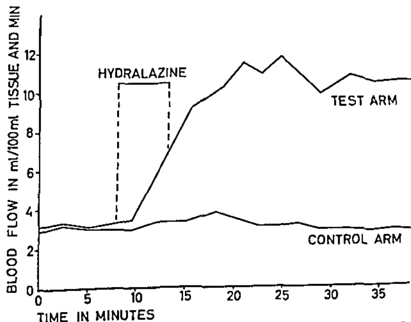


Fig 1 Blood flow in upper part of the forearm before and after infusion of hydralazine (total dose 0 36 mg) into one brachial artery

Table 2

Effect of intra arterially injected hydralazine on blood flow in the upper part of the forearms. The drug was injected into the brachial artery of the test arm during 15 seconds. Blood flow in ml per 100 ml tissue per minute

Exp No	Dose of hydralazine mg	Forearm volume ml		Before injection		After injection of hydralazine: 1 6 min		7 12 min		13 18 min		19 24 min		25 30 min		55 60 min		4 hours	
		Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm
7	0.27	665	675	3.4	2.7	4.5	3.1	5.8	3.0	6.3	3.1	6.5	2.8	6.8	2.9	6.6	2.8	5.0	3.1
8	0.19	585	585	2.4	1.9	5.1	2.5	5.8	2.6	6.7	2.5	8.5	2.9	8.3	2.8	7.8	3.1	2.4	2.5
9	0.27	585	585	4.2	5.6	6.8	6.2	8.7	6.7	8.3	4.7	8.9	4.8	9.7	5.8	9.0	5.5	7.7	5.8
10	0.27	705	640	2.6	2.3	3.7	2.2	4.5	2.0	5.5	2.8	4.6	1.9	4.6	2.0	4.3	1.9	3.3	2.3
11	0.23	555	515	1.7	2.9	5.2	3.0	6.2	2.9	6.1	2.9	6.0	3.0	6.1	3.2	5.1	3.5	5.0	4.3
Mean values	0.25	619	600	3.26	3.08	5.06	3.40	6.20	3.44	6.58	3.20	6.90	3.08	7.10	3.34	6.56	3.36	4.68	3.60
Mean change in test arm after hydralazine						1.80 ±0.35[2]		2.94 ±0.46[2]		3.32 ±0.37[3]		3.64 ±0.77[2]		3.84 ±0.82[2]		3.30 ±0.80[1]		1.42 ±0.59	
Mean change in control arm after hydralazine							0.32 ±0.14		0.36 ±0.25		0.12 ±0.21		0.00 ±0.30		0.26 ±0.18		0.28 ±0.19		0.52 ±0.24
Mean difference between test arm and control arm				0.18 ±0.36		1.66 ±0.35[2]		2.76 ±0.24[3]		3.38 ±0.25[3]		3.82 ±0.56[2]		3.76 ±0.51[2]		3.20 ±0.54[2]		1.08 ±0.38[1]	

[1] $P < 0.05$
[2] $P < 0.01$
[3] $P < 0.001$

The effects of intra-arterially administrated hydralazine (hydralazine chloride, apresoline ®, Ciba) on the blood-flow in the arms were studied in three series of experiments In the first, the drug was infused for five minutes and the circulatory response in the forearms was recorded for at least 30 minutes In the second run the drug was given by rapid injection (duration 15 seconds) and its effects on the forearm blood flow were recorded continuously for about 60 minutes; the catheter was then removed, and the experiment was interrupted, but about four hours after the injection the plethysmographs were again applied and the blood flow measured for about fifteen minutes In the third series the drug was infused for five minutes and the effects on the bloodflow of the hands were recorded for at least 70 minutes

For statistical analysis of the results the *t* test (FISHER 1936) was employed

Results.

1 *Effects of Intra arterially Infused Hydralazine on the Blood Flow in the Upper Part of the Forearm*

A typical experiment is illustrated in figure 1, and the results of six experiments are given in table 1 Hydralazine was infused in a total dose of 0 18–0 36 mg It increased the blood flow in the forearm of the infused limb (test arm) The effect set in after a latency of one to two minutes and reached its maximum approximately 12 minutes after beginning infusion On the average the blood flow in the test arms rose from 4 57 ml/100 ml tissue/minute (simplified, in the following, as "ml") before the infusion, to 8 23 ml 13–18 minutes after the start of infusion (difference 3 66 ± 0 92 ml, P < 0 02) The effect thereafter remained unchanged until the experiment was ended 30 minutes after beginning infusion The blood flow in the contralateral arm (control arm) showed no significant change after the administration of hydralazine – In three experiments blood pressure and pulse rate were taken before and 30 minutes after the infusion, and no appreciable change was observed

2 *Effects of Intra-arterially Injected Hydralazine on the Blood Flow in the Upper Part of the Forearms*

Table 2 gives the results of five experiments The effect of hydralazine (0 19–0 27 mg) on the blood flow in the test arms was slow in onset Compared with the pre-injection value the blood flow was, on the average, 1 80 ml higher for the first six-minute period (± 0 35, P < 0 01), 2 94 ml for the second (± 0 46, P < 0 01), 3 32 ml for the third (± 0 37, P < 0 001); and 3 64 for the fourth six-minute period, 19–24 minutes after the

Table 3

The effects of intra arterially infused hydralazine on blood flow in the hands. The drug was infused into the brachial artery of the test arm for five minutes. Blood flow in ml per 100 ml tissue per minute

Exp No	Dose of hydralazine mg	Hand volume ml		Before infusion		After start of infusion of hydralazine											
						1–6 min		7–12 min		13–18 min		19–24 min		25–30 min		About 70 min	
		Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand
12	0.47	420	475	21.2	23.8	24.8	19.2	29.6	18.6	28.5	12.9	31.0	16.2	32.9	16.0	24.2	18.8
13	0.31	470	473	7.6	9.6	12.8	10.0	15.7	9.9	16.4	8.8	21.6	11.2	25.3	15.3	21.1	11.7
14	0.55	420	455	20.4	21.1	26.9	26.7	34.2	26.0	34.6	20.8	37.1	21.2	36.9	22.9	26.5	14.8
15	0.47	490	435	33.4	33.0	33.2	30.4	40.5	33.8	40.2	33.6	38.2	29.2	39.6	29.8	31.3	26.9
16	0.47	465	480	8.8	8.7	10.0	9.3	14.5	6.7	17.8	7.9	17.1	7.0	17.5	11.3	15.8	7.4
17	0.39	430	415	15.6	15.4	20.9	18.8	26.4	16.9	24.2	16.1	31.9	18.3	26.6	18.9	33.1	24.7
Mean values	0.44	449	456	17.83	18.93	21.43	19.07	26.82	18.65	26.95	16.68	29.48	17.18	29.80	19.03	25.33	17.38
Mean change in test hand after hydralazine						3.60 ±1.07[1]		8.99 ±1.18[1]		9.12 ±1.08[3]		11.65 ±1.95[2]		11.97 ±1.81[2]		7.50 ±2.89[1]	
Mean change in control hand after hydralazine							0.14 ±1.33		−0.28 ±1.18		−2.25 ±1.79		−1.75 ±1.54		0.10 ±2.02		−1.55 ±1.99
Mean difference between test hand and control hand				1.10 ±0.61		2.36 ±0.78[1]		8.17 ±0.77[3]		10.27 ±1.49[3]		12.30 ±1.16[3]		10.77 ±1.63[3]		7.95 ±1.09[3]	

1) $P < 0.05$
2) $P < 0.01$
3) $P < 0.001$

injection (± 0 77, P < 0 01) One hour after the injection the effect remained largely unchanged Four hours after the injection a distinct effect persisted in two arms, on the average, the blood flow in the five test arms was 1 08 ml greater than that in the control arms (± 0 38, P < 0 05) No significant changes of blood flow in the control arms were recordable during the first hour after the injection

3 *Effects of Intra-arterially Infused Hydralazine on the Blood Flow in the Hands*

These effects were studied in six experiments (table 3) Hydralazine (total dose, 0 31–0 55 mg) produced an increase of blood flow in the test hands Here also the effect occurred slowly and reached its maximum approximately 20 minutes after beginning infusion The blood flow averaged 17 83 ml before the infusion and 29 48 ml 19–24 minutes after it was begun (difference 11 65 ± 1 95 ml, P < 0 01) About 70 minutes after the infusion the effect was still persistent, the blood flow being 7 50 ml higher than the preinfusion value (± 2 89, P < 0 05) In the control hands the infusion produced no significant alteration in blood flow

Discussion.

Hydralazine administered in the brachial artery depressed the peripheral vascular resistance in the ipsilateral forearm and hand without producing systemic effects If the whole of the intra-arterial dose of hydralazine had left the blood stream during transit through the arm, the substance would have had a concentration of approximately 0 2–0 4 mg/kg in the arm tissues When administered intravenously to anaesthetized animals hydralazine has a hypotensive effect in doses down to 0 1 mg/kg (BEIN *et al* 1953), and in most studies of its haemodynamic action in man intravenous doses of 0 2–0 5 mg/kg have been used In our experiments therefore, the tissue concentration of hydralazine in the forearm and hand was probably within the range that may be attained on intravenous administration of the drug in therapeutic doses – The vasodilator action in the forearm and hand was slow in onset, and the time course was similar to that of the hypotensive action after intravenous administration (ESSELIER *et al* 1953)

Our results are consistant with previously reported experimental observations, which have shown that in human subjects the hypotensive effect of hydralazine is largely attributable to its peripheral site of action in the vascular bed (STUNKARD *et al* 1954, ÅBLAD 1959)

Since the blood flow response to hydralazine in the forearm and hand persisted for a considerable time after administering the drug, this appears

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Normorphine, Nalorphine and Morphine. Quantitative Separation and Determination of Small Amounts in Blood and Tissues.

By

Kirsten Milthers

(Received May 8 1961)

It has generally been assumed that morphine is N demethylated to normorphine in the organism This is what happens, for instance, with codeine (ADLER *et al* 1955) Thus, though normorphine has been established as a morphine metabolite *in vitro* (AXELROD 1956), evidence for its formation *in vivo* is indirect The only experimental evidence available at present suggesting normorphine formation *in vivo* is the observation that $C^{14}O_2$ appears in the breath shortly after injection of morphine-N $C^{14}H_3$ into rats (MARCH & ELLIOT 1954) or man (ELLIOT *et al* 1954) Obviously such tracer experiments give no more than indirect evidence for the formation of normorphine as a metabolite of morphine It was therefore thought important to elaborate methods permitting the quantitative determination of both normorphine and morphine in various tissues

BECKETT CASY & HARPER (1956) have postulated that the nor-derivative of morphine is responsible for the analgesic effect This hypothesis and the confirmed antagonism of nalorphine to morphine analgesia indicate the importance of also determining nalorphine by the same procedure

Methods for quantitatively determining small amounts of *morphine* in blood and tissues have been described previously (MILTHERS 1958, 1959)

Methods for determining *normorphine* have not been found in the literature but a method for separating morphine and normorphine by paper chromatography has been put forward previously (MILTHERS 1959)

The determination of *nalorphine* had been described by WOODS & MUEHLENBECK (1957) and its separation from morphine by paper chromatography by PEDLEY (1955) Here we present a method by which it is

to have been relatively firmly attached to its site of action A similar finding has emerged from *in vitro* studies of bovine mesenteric artery preparations In such preparations hydralazine lessened the effect of various tonus-increasing stimuli, and its action persisted for two hours after it had been washed out of the bath (ÅBLAD, unpublished results)

Summary.

Hydralazine was administered unilaterally into a brachial artery in man the dose being such that the tissue concentration of the drug in the arm was presumptively within the range associated with systemic administration of therapeutic doses It substantially increased the blood flow in the proximal part of the forearm and in the hand, whereas the blood flow in the contralateral arm was unchanged The local vasodilatation developed slowly, reached its maximum 12–20 minutes after administering the drug and persisted for more than one hour The results lend support to the view that the hypotensive effect of hydralazine is due largely to its peripheral site of action in the vascular bed

Acknowledgement

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113

overies of nalorphine and morphine were still approximately 100% We did not succeed in obtaining higher recoveries of normorphine by application of other extraction mixtures This is most probably due to the low solubility of the normorphine in all solvents

To avoid time-wasting titrations, some further investigations were carried out We found, that on adding 4 g sodium carbonate to the 5 ml

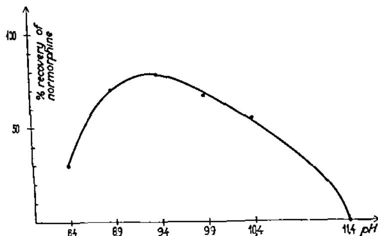


Fig 1 Percentage recovery of normorphine
The normorphine is extracted from aqueous solutions at different pH values three times with equal volumes of a mixture of chloroform and *iso*-propanol (3 1)

in the separation funnel just before the extraction, there resulted a pH a little higher, as well as a higher recovery, presumably on account of a salting out effect

Paper Chromatography

Separation of normorphine, nalorphine and morphine was accomplished by paper chromatography The main difficulty lies in separating normorphine from morphine, because the R_F values of these two substances are close to one another in most chromatographic mixtures As mentioned previously (MILTHERS 1959) this separation was carried through by descending paper chromatography with the chromatographic mixture amylene hydrate, *di n* buthylether and water, 80 7 13 (BROSSIE, HAFLIGER & SCHNIDER 1955) We found that for this mixture the R_F values for normorphine, nalorphine and morphine were 0 04, 0 50 and 0 08, respectively

The procedure was, with some modifications, the same as that described by PÆRREGAARD (1957)

possible, after extraction of tissue-homogenates and subsequent chromatographic separation, to determine normorphine, nalorphine and morphine polarographically in various tissues, at concentrations down to 0.6 µg/g with a recovery of 72%, 97%, and 97%, respectively

Preliminary Investigations.

Polarography

The last stage in the method of morphine determination previously described by the author is the polarographic determination

Morphine as such cannot be determined polarographically, but it is transformed after treatment with nitrite in acid solution into a derivative that can be determined polarografically This derivative has been described earlier as a nitroso derivative, but recently it has been shown to be a nitro derivative (BAGGESGAARD-RASMUSSEN & BOLL 1958, LUND 1958)

Normorphine and nalorphine can both be determined polarographically after treatment with nitrite in acid solution, having a half-wave potential about 1.0 v, similarly to morphine The procedure is the same as described by PÆRREGAARD (1957) There is proportionality between the concentration of morphine, as also of that of normorphine and nalorphine, and the wave-height at maximum sensitivity (i e observed wave-height in mm multiplied by the reciprocal of the sensitivity of the apparatus on which the curve is obtained) This proportionality is valid for concentrations up to 500 µg/ml and probably for still higher concentrations The average ratio between the morphine concentration expressed in µg/ml and the calculated wave-height at maximum sensitivity expressed in mm is 0.0352 For normorphine and nalorphine the average ratios are 0.0270 and 0.0316, respectively The sensitivity of the polarograph was controlled every day by means of standard solutions before and after use

Extraction

By the method previously referred to, the homogenates were deproteinised with uranyl chloride The supernatant liquid was autoclaved and the morphine extracted at bicarbonate-alkaline reaction (i e at pH about 8.4) three times with equal volumes of a mixture of chloroform and *iso*-propanol (3:1) By this procedure nalorphine was also extracted quantitatively, but only 30–40% of the normorphine The isoelectric point of morphine is about pH 9, and one might expect it to be higher for normorphine, a secondary amine By extraction at different pH-values, adjusted by titration with sodium carbonate, we found the best recovery to occur at about pH 9.4 (see fig 1) The average percentage recovery of normorphine was there about 78% from aqueous solutions The rec-

are more compact The sheet was then cut off just above the zones corresponding to

& MARTIN (1947)

The excised piece of paper was cut to a point at one end, and the other end was placed on the free end of a double wick (4 × 6 cm) of Whatman paper No 1, which

sucked through the wick and the piece of paper down into the pipette, took the nalorphine along with it The elution volume was from 0 50–1 00 ml, and N HCl was added to give a total volume of 1 00 ml

The lower edge of the remaining part of the sheet bearing the spots of normorphine and morphine was then serrated, and paper chromatography was continued in the same chromatographic vessel after resaturation of the sheet After the paper chromatography had continued for some further 28 hours (total about 45 hours), the sheet was removed again from the vessel and dried Localisation and the elution of normorphine and morphine were carried out as for nalorphine

In our preliminary investigations we encountered a problem which we strongly should advise anybody to take care of In the chromatographic mixture we use *di-n*-buthylether, and this ether *often contains peroxides* even when of analytical reagent quality, because they develop on storage Morphine, normorphine, nalorphine and other substances are destroyed by peroxides We have noted a loss of up to 40%, even after paper chromatography for only 2 hours, if the *di n*-buthylether contained peroxides The peroxides can be removed by washing the ether several times with ferrous sulphate and then with water, and finally removing the water by shaking with potassium hydroxide pellets Development of peroxides is avoided by storage in the dark at 4°C with potassium hydroxide pellets

Separation and Quantitative Determination of Normorphine, Nalorphine and Morphine in Tissues.

The substances used in the experiments were normorphine hydrochloride ($C_{16}H_{17}O_3N$ HCl H_2O) melting point 305 –307°, obtained from the Department of Health Education and Welfare, *U S A*, nalorphine hydrochloride ($C_{19}H_{21}O_3N$ HCl) (anarcon ℞) GEA Pharmaceutical Products, and morphine hydrochloride ($C_{17}H_{19}O_3N$ HCl $3H_2O$) Ph Dan 1948

Technique

The experiments were carried out with organs freshly removed from normal adult male rats, except for a few experiments on normal adult male rabbits

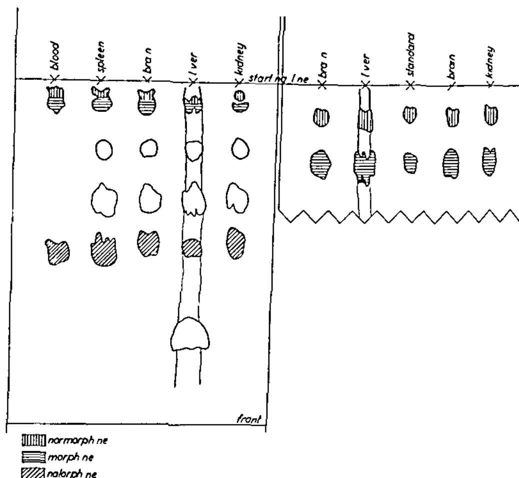


Fig 2 Two stages in the separation of nalorphine normorphine and morphine by paper chromatography

The left part of the figure shows the spots of normorphine morphine and nalorphine after chromatography for 17 hours nalorphine is clearly separated from morphine and normorphine

The right part of the figure shows the remaining part of the sheet when it has been cut off just above the zone corresponding to the nalorphine spots after 17 hours of chromatography The lower end of the sheet has been serrated and chromatography continued for a further 28 hours At this time (after 45 hours chromatography) normorphine has been separated from morphine

The paper used was Whatman No 1 buffered at pH 6 3 The paper was first drawn twice through an aqueous buffer solution consisting of 15 6 g NaH_2PO_4 2 H_2O and 3 5 ml of 30% w/v NaOH per 1000 ml solution and then dried Each sheet measured 50 × 23 cm A transverse starting line was drawn 12 cm from the upper end and five samples and a standard were applied The sheet was then suspended for 3 hours saturation in the chromatographic vessel (20 × 30 × 50 cm) Descending chromatography was begun, when the fluid level approached the lower end of the paper after 16–18 hours at 22°C, the paper was removed from the vessel and dried The nalorphine was localized in ultraviolet light by means of absorption from the standard spot The absorptions in ultraviolet light are marked with amounts of nalorphine down to 10 μg and with amounts of morphine and normorphine down to 2 μg because these two substances only run a short distance on the sheet and consequently the spots

are more compact The sheet was then cut off just above the zones corresponding to the nalorphine spot, usually 12–14 cm from the origin The zones were cut out of the other five lengths, and each of these five pieces of paper was eluted separately Elution was carried out as described by PÆRREGAARD with the technique of CONSDEN, GORDON & MARTIN (1947)

The excised piece of paper was cut to a point at one end, and the other end was placed on the free end of a double wick (4 × 6 cm) of Whatman paper No 1, which was placed over the edge of a vessel containing N HCl The tapering end of the paper carrying the nalorphine was brought into contact with the point of a graduated cylindrical pipette of 1 00 ml capacity The hydrochloric acid in the vessel, on being sucked through the wick and the piece of paper down into the pipette, took the nalorphine along with it The elution volume was from 0 50–1 00 ml, and N-HCl was added to give a total volume of 1 00 ml

The lower edge of the remaining part of the sheet bearing the spots of normorphine and morphine was then serrated and paper chromatography was continued in the same chromatographic vessel after resaturation of the sheet After the paper chromatography had continued for some further 28 hours (total about 45 hours), the sheet was removed again from the vessel and dried Localisation and the elution of normorphine and morphine were carried out as for nalorphine

In our preliminary investigations we encountered a problem which we strongly should advise anybody to take care of In the chromatographic mixture we use *di n*-buthylether, and this ether *often contains peroxides* even when of analytical reagent quality, because they develop on storage Morphine, normorphine, nalorphine and other substances are destroyed by peroxides We have noted a loss of up to 40%, even after paper chromatography for only 2 hours, if the *di n* buthylether contained peroxides The peroxides can be removed by washing the ether several times with ferrous sulphate and then with water, and finally removing the water by shaking with potassium hydroxide pellets Development of peroxides is avoided by storage in the dark at 4°C with potassium hydroxide pellets

Separation and Quantitative Determination of Normorphine, Nalorphine and Morphine in Tissues.

The *substances used* in the experiments were normorphine hydrochloride ($C_{16}H_{17}O_3N$ HCl H_2O) melting point 305 –307°, obtained from the *Department of Health* Education and Welfare U S A , nalorphine hydrochloride ($C_{19}H_{21}O_3N$ HCl) (anarcon ®) GEA Pharmaceutical Products and morphine hydrochloride (C_1 $H_{19}O_3N$ HCl $3H_2O$) Ph Dan 1948

Technique

The experiments were carried out with organs freshly removed from normal adult male rats, except for a few experiments on normal adult male rabbits

The organs were homogenised in a microhomogeniser (Potter & Elvehjem) with 0 9% w/v sodium chloride solution The concentrations of homogenates in our experiments ranged from 20% to 50% w/v, depending on the weight of the organs

To homogenate samples of 4 25 ml were added 250 µl of aqueous solutions of normorphine, nalorphine and morphine, so that *each* sample contained 10, 20, 40, 60, or 100 µg of the three substances per 5 ml homogenate Of these homogenates 5 00 ml were deproteinised with uranyl chloride, as described for morphine determination in blood (MILTHERS 1958) The supernatant liquid was drawn off, 10% 10N-HCl was added, and the mixture was autoclaved at 120° C for 30 minutes To 5 00 ml of the solution were added 4 g sodium carbonate, and this was then extracted three times with equal volumes of a mixture of chloroform and *iso* propanol (3 1) The extracts were filtered through anhydrous sodium sulphate and after evaporation to dryness the residues were dissolved in 100 µl of a mixture of chloroform and methanol (2 1) and applied to the Whatman paper Washing was performed twice with 100 µl of chloroform methanol, applied at the same origin as the first 100 µl Paper chromatography was then carried out as described above After 16–18 hours of chromatography the paper was dried, the sheet cut off just above the localized nalorphine zone and the lower edge serrated Chromatography was begun again and carried on for some further 28 hours, to ensure the separation of normorphine from morphine The paper with the nalorphine spots was cut out and eluted as described above To the eluate was added N-HCl to a total volume of 1 00 ml, and this was treated with 400 µl M potassium nitrite for 5 minutes The reaction was stopped by adding 600 µl 20% w/v potassium hydroxide, and the nalorphine was determined polarographically After a total period of about 45 hours of chromatography, normorphine and morphine were localized, cut out, eluted an determined polarographically

Results.

As seen in table 1 the percentage recoveries of added normorphine, nalorphine and morphine were approximately 72%, 97% and 97% respectively The individual results showed variations up to ± 6%

The sensitivity is to a certain extent limited by the sensitivity of the polarographic determination, which requires not less than 3–5 µg of these substances, in other words, by the standard method with 5 ml homogenate it is possible to determine 2–3 µg/g tissues Greater sensitivity can be

Table 1

Recovery after separation of normorphine, nalorphine and morphine in tissues containing known added amounts of the compounds

Added compounds µg/5 ml homogenate		10	20	40	60	100	Average recovery per cent
Blood undiluted	normorphine	7 92	14 0	27 2	46 8	79 5	75
	nalorphine	9 58	19 1	39 3	57 2	101	97
	morphine	8 78	19 4	38 7	58 8	100	96
Brain 25%[1])	normorphine	7 25	14 8	25 4	40 1	81 1	72
	nalorphine	9 65	19 4	39 9	55 6	97 2	97
	morphine	9 78	18 9	40 0	57 8	97 5	97
Liver 50%[1])	normorphine	7 04	12 5	24 6	39 7	68 3	66
	nalorphine	9 01	19 4	40 1	59 8	95 3	96
	morphine	9 98	20 6	40 5	61 1	99 6	101
Spleen 20%[1])	normorphine	7 41	14 7	28 1	41 8	74 3	72
	nalorphine	9 56	19 8	40 8	57 8	100	99
	morphine	9 13	19 2	40 5	58 3	97 9	97
Kidney 33%[1])	normorphine	6 67	14 5	29 0	46 0	75 6	73
	nalorphine	9 78	19 0	40 7	56 7	93 1	97
	morphine	9 60	18 9	39 4	56 8	98 3	97

[1]) Per cent tissue in the homogenate (w/v)

obtained by using a larger volume of homogenate We have determined as little as 0 6 µg/g tissues in 20 ml homogenate 50% w/v without reducing the accuracy

Summary.

A method is described for simultaneous determination of normorphine, nalorphine and morphine in tissues The homogenate is deproteinised, autoclaved in acid solution and extracted at alkaline reaction with a mixture of chloroform and *iso* propanol The substances are then separated by descending paper chromatography and finally determined polarographically

By this method it is possible to determine as small as 2 5 µg/g tissue on 5 ml homogenate 50% and 0 6 µg/g on 20 ml homogenate 50%, with a recovery of 72 ± 6% for normorphine and 97 ± 6% for both nalorphine and morphine

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The Combined Effect of Adrenaline and Sodium Chloride on Blood Pressure in Normal Rats

By

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Emotional factors as for instance mental exertion, psychic strain and agressive states increase the blood pressure (MILLS 1959, WOLFF 1953, PICKERING 1955) These factors also increase the excretion of adrenaline (GOODALL & MEEHAN 1956 ZUIDEMA *et al* 1956) e g during matriculation examinations to fourfold (PEKKARINEN *et al* 1959) Audiogenic and visual stimuli can cause transient hypertension in halfwild rats (FARRIS *et al* 1945, ROTHLIN *et al* 1955) especially old ones (MEDOFF & BONGIOVANNI 1945)

An adrenaline infusion decreases renal blood flow (GOLDZIEHER 1946, HARTMAN & BROWNELL 1949) Electric stimulation of the sympathetic renal nerves (GOODWIN *et al* 1949) and the cerebral cortex (HOFF *et al* 1949) as also intravenous injection of adrenaline (TRUETA *et al* 1947, CERLETTI *et al* 1949) cause an experimental ischaemia of the renal cortex Psychic factors can decrease renal blood flow to ½ of its normal value even though blood flow in other organs remains normal (WOLFF 1953, SCHROEDER 1957) The renal vasoconstriction can last longer than the rise in blood pressure especially in essential hypertension (WOLFF 1953)

In man pheochromocytoma can cause all the characteristic signs of hypertension in the blood vessels there may appear diffuse, degenerative and partly necrotic changes like those after prolonged administration of adrenaline (RAAB 1953 ENGER 1940) This increases the blood pressure in the dog to some degree (LANGE 1924, ENGER 1940) but the effect is slightly clearer in the rat (HEIM 1952) Adrenaline and noradrenaline cause a marked rise in blood pressure during experimental renal hypertension (OLSEN *et al* 1950)

Sodium chloride solution in place of drinking water or addition salt to the diet increases the blood pressure (SAPIRSTEIN *et al* 1950,

PICKERING 1955; SELYE 1955–56), as do other sensitising factors The rise in the blood pressure of experimental animals depends on the daily intake of sodium chloride (MENEELY *et al.* 1953). Essential hypertension is rare in individuals on a low salt intake (DAHL 1959) DOCA enhances and lack of sodium chloride decreases the effect of adrenaline or noradrenaline injections in acute test (RAAB 1953, 1959)

The doses of DOCA needed to increase blood pressure (2 5 mg daily) are, however, relatively big (KNOWLTON *et al.* 1946; PICKERING 1955) Salt is an essential factor also in the development of hypertension caused by large aldosterone doses (0.5 mg daily) in rats sensitised by unilateral nephrectomy (GROSS *et al.* 1957). Because the importance of salt in the experimental of hypertension, and also the vasocontrictor effects of the catecholamines in acute tests, we decided to study the combined effect of sodium chloride and prolonged adrenaline in chronic tests

Materials and Methods.

The material included 105 normal albino rats, 69 males and 36 females Their average initial weight was 282 g In the adrenaline + sodium chloride group was 35 rats, in the sodium chloride group 34, in the adrenaline group 8 and in the control group 8 In the acute test there were ten rats in each two groups (adrenaline or adrenaline along with salt)

The single dose of adrenaline in oil was 1 mg (Adrenalin Retard, Leo, dissolved in 0 2 ml of oil solution containing ascorbic acid 0 015, white beeswax 0 02, anhydrous lanoline 0 04 and arachis oil to 1 ml) The adrenaline in oil was given as a daily injection into the dorsal regions subcutaneously near the median line in all about 3000 injections were given Sodium chloride was administered in the drinking water as 1–2% The measurements of blood pressure (once in 7–14 days) were carried out 20–24 hours after the adrenaline injection with photoelectric tensometer from the hind leg without anaesthesia (Metro Industries) Every blood pressure measurement was checked at least three times Before the blood pressure measurement the rats were kept in an animal holder for about 5–10 minutes, after which they generally remained sedated during the measuring time

Statistical treatment Student's p-test was used for comparing blood pressures The result was considered significant at $P < 0\ 01$

Results.

Bodyweight (fig 1) The average initial bodyweight was in the adrenaline + sodium chloride group 320 g; in the adrenaline group 237 g, in the sodium chloride group 290 g; and in the control group 256 g An increase in body weight was observed only in the control group In the other groups the changes of body weight were small

The course of the experiment. At ½–1 hour after the adrenaline injections the rats were obviously tired and weak, and they were breathing rapidly

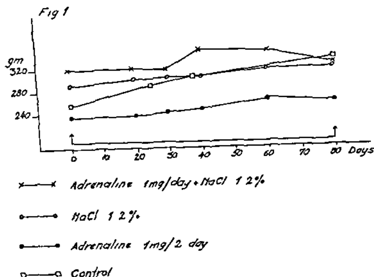


Fig 1 Average body weights of the rats in the control group and during prolonged treatment with adrenaline and sodium chloride

and panting About 1½–2 hours later these effects had disappeared, and the rats seemed normal again At the end of the treatment almost all the test animals showed a tendency to local infections in the skin of the dorsal region and back, though these seemed to have little effect on their general condition The mortality in both control and adrenaline groups was nil When the tests in the adrenaline + sodium chloride group had lasted 30 days 10% of the rats were dead, and at the end of the experiment 31% At autopsy some of the dead animals had extensive pneumonia like purulent infections Increased nervousness and agressiveness were seen more noticeably in the adrenaline + sodium chloride group than in any other

A *Acute Test*

One single injection of adrenaline in oil (1 mg s c) caused a rise in blood pressure from 106 to 121 mm Hg in normal rats (fig 2) In the group of rats that for two weeks had had 2% sodium chloride in the diet, the adrenaline in oil caused a rise in blood pressure from 116 to 135 mm Hg In both groups the increase in blood pressure lasted about 6 hours

B *The Chronic Test*

In the adrenaline + sodium chloride group (fig 3 A) (Adrenaline 1 mg/day s c = about 3–4 mg/kg and 1–2% sodium chloride as drinking

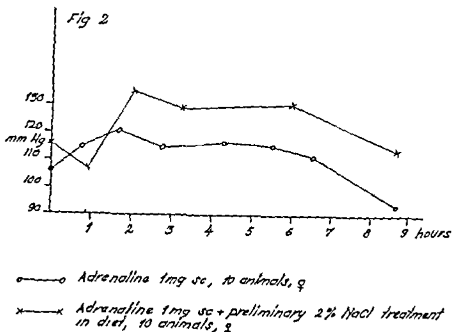


Fig 2 Effect of adrenaline in oil (1 mg subcutaneously) on the blood pressure in the control group and in the group after 2 weeks' treatment with 2 % sodium chloride in the diet Each value is the average for ten rats

water) there was a tendency to increase the blood pressure already after a week (from 114 to 125 mm Hg) The blood pressure then increased continuously, reaching a maximum (146 mm Hg) on about the 50th day After that the pressure began to decrease gradually, despite daily adrenaline injections After 70 days the blood pressure decreased quickly, in about 20 days, to 10 mm Hg above the original level

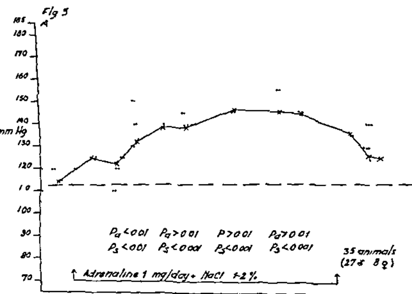
In the sodium chloride group (fig 3 B) the blood pressure in 20 days increased to 125 mm Hg, after which it increased much more slowly The peak (130 mm Hg) was reached on the 75th day After the salt treatment the blood pressure fell in a few days, but not reached yet the level in the beginning of experiment

In the adrenaline group (fig 4 A) the rise in blood pressure was slow (118 mm Hg after 27 days), it then became more pronounced and continued until the 56th day The blood pressure had then reached its maximum (136 mm Hg) and clearly began to fall from the 80th day onwards

The animals *in the control group* (fig 4 B) were given the same food as the other groups The drinking water was tap water Their average blood pressure was observed to rise only slightly for 110 days, from 110 to 117 mm Hg

C *Comparison of the Adrenaline + Sodium Chloride and the Sodium Chloride Groups in the Chronic Test*

On comparing the adrenaline + sodium chloride group with the sodium chloride group, it will be noticed that the rise in the blood pressure



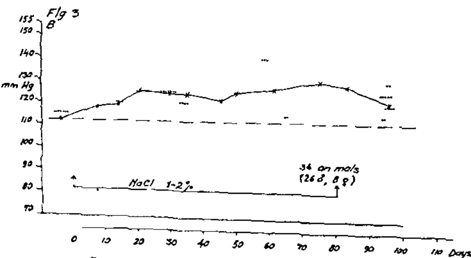


Fig 3 Effect of prolonged treatment with adrenaline in oil and 1 2 % sodium chloride solution on the blood pressure of rats Individual values and means The average normal blood pressure broken line
A = Adrenal ne 1 m ... % sodium chloric
B = ...
P_a ...
P_s = ...

is clearly ...
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... group The difference was clear after 18–20

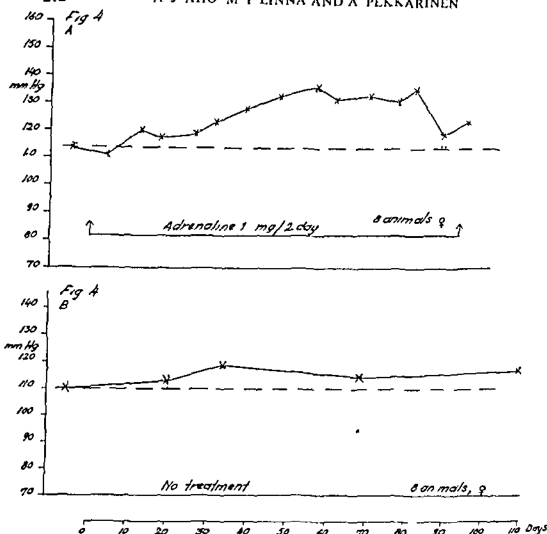


Fig 4 Effect of prolonged treatment with adrenaline in oil on the blood pressure of rats
Individual values and the means The average normal blood pressure broken line
A = Adrenaline 1 mg subcutaneously every other day
B — Control blood pressure for the same time

days, when 69% in the adrenaline + salt group had a blood pressure of ≦ 130 mm Hg, the corresponding value for the salt group being 37 5% (Fig 5) The clearest difference in blood pressures was on the 47–50th days, when values of ≤ 130 mm Hg were found in 85% of the adrenaline + salt group and in 21% of the salt group and values of ≦ 150 mm Hg in 41% of the former and 7% of the latter group At the end of the test blood pressures were found to be considerably higher in the adrenaline + salt than in the salt group This is also illustrated by the fact that values of ≦ 170 mm Hg were found only in the adrenaline + salt group

Comparison of adrenaline + sodium chloride and adrenaline groups When the adrenaline + salt group and the adrenaline group are compared

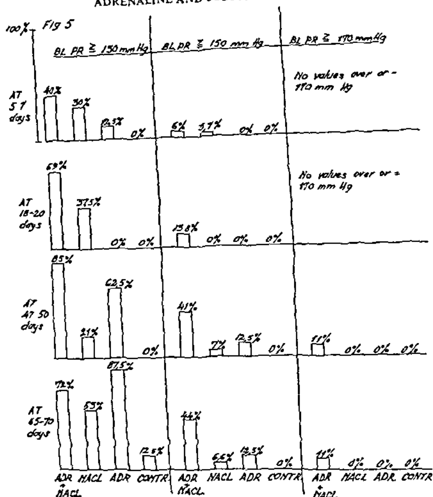


Fig 5 Percentage distribution of individual values for blood pressure during prolonged treatment with adrenaline injections and sodium chloride as well as for the sodium chloride adrenaline and control groups

higher average values of blood pressure appeared in the former group (fig 4 A and fig 5), but the difference was not significant at the 0 05 level Analysis showed that the difference in blood pressure between the groups became significant only on the 20th test day ($P < 0.01$) (Fig 3A)

Discussion.

The combined effect of adrenaline and sodium chloride on blood pressure in our experiments to some extent supports opinion about the role of sodium chloride retention and increased secretion of adrenergic

substances in the aetiology of hypertension *Sodium retention, especially in the smooth muscles of the blood vessels, may enhance the effects of the vasoconstrictor impulses of the sympathetic nerve fibres* (RAAB 1953) A normal sympathetic tone can already then cause vasospasm, and an increased vasomotor tone as well as noradrenaline raise it considerably (SCHROEDER 1957) According to CROUT (1959) (RAAB 1959) a number of studies suggest that vascular reactivity to catecholamines may be increased in hypertension

It has been stated that an adrenaline injection decreases renal blood flow (GOLDZIEHER 1946, HARTMAN & BROWNELL 1949) and causes ischaemia in the renal cortex in animal experiments (TRUETA *et al* 1947, CERLETTI *et al* 1949) It has also been shown that psychic stimuli produce renal vasoconstriction in man (WOLFF 1953) These effects may be basic to the development of increased blood pressure from prolonged adrenaline and sodium chloride treatment

Repeated administration of adrenaline in oil (2–4 mg kg) or in water (0 4–1 7 mg/kg) to rats has been shown to cause transient hypertensive reactions (140 mm Hg), beginning on 5–7th day during prolonged treatment for 130 days (HEIM 1952) These reactions tend to become normal later on, despite the treatment The blood pressure measurements were performed with the plethysmograph under ether anaesthesia For shorter periods, 1–14 days, 1 mg adrenaline in oil, causes hypertension in rats only occasionally (SCHROEDER & NEUMAN 1942) In dogs daily injections of 1–6 mg (up to 40 mg) adrenaline in aqueous solution, for 8 months to 2 years, elevated the systolic blood pressure, in one dog maximally by 90 mm Hg in 3 months Later the blood pressure began to return to normal in spite of continued injections (ENGER 1940)

In our experiments the effect of adrenaline alone upon the blood pressure in a rat was slight (fig 4 A) We did not observe as high (180 mm Hg) *individual blood pressure values as did Heim* although some raised values (145–155 mm Hg) were in fact seen in our rats after the 55th day In our *preliminary tests series the injections of adrenaline in oil increased the* blood pressure in old rats up to 160–170 mm Hg (AHO *et al* 1959) in 2½ *months So great a rise we have, however, not been able to confirm in* later experiments The age of the animals may be of importance, e g the *audiogenic stimuli increase the blood pressure more in old than in young* rats (MEDOFF & BONGIOVANNI 1945)

In our experiments the injections of adrenaline in oil were considered
sympathetic tone Clearly, the effect
e same as those of the endogenic
and adrenaline, upon the receptors

In spite of the fact that adrenaline in the doses used produced a shocklike

state of fatigue in rats for some hours, the vasopressor effect in the acute experiment was small

The adrenaline dose, 1 mg daily, is large per unit of body weight, compared e g, with the doses used clinically The rat is insensitive to adrenaline The effect in the acute test lasted only 5–6 hours (fig 2) The test animals, e g the guinea pig and the monkey, require rather high doses of adrenaline to produce even a short rise in blood pressure (KASAHARA & KAWAMURA 1937)

The level and the increase of blood pressure in experimental studies depend on the amount of sodium chloride in the diet (MENEELY *et al* 1953) These workers gave the rats a sodium chloride diet for over one year, the salt content ranging from 0 01 to 9 8% Prolonged administration of 2 8–5 6% sodium chloride in the diet increased the blood pressure to a slightly hypertensive level during 9 months treatment 2 8% sodium chloride in the diet of the test animals corresponds to a daily consumption of 14 g salt for a 70 kg man (MENEELY 1959) 1–2% sodium chloride in drinking water, as used by us, about corresponds to Meneely's 2 8% diet, since in our test the salt content of the food increased the total amount of sodium chloride Drinking water containing 2% sodium chloride caused hypertension in all rats (BRANDT *et al* 1951) after six weeks, and the blood pressure then returned to normal

Two common physiological effects in normal metabolism, those of adrenaline and sodium chloride, when combined, in our experiments increased the blood pressure of rats The combined effect of these factors has not been previously studied in chronic tests Several investigators emphasize the importance of sodium chloride and psychic factors in the development of hypertension The response of the blood pressure in rats during prolonged treatment with adrenaline in oil and sodium chloride is to a large extent individual In our own experiments there were some animals in which the blood pressure reached the hypertensive level About the individual variation of the blood pressure values in test animals there are also earlier findings in rats (HEIM 1952) and dogs (SCHROEDER & GOLDMAN 1952) during prolonged treatment with adrenaline only

Summary.

The combined effect upon blood pres[illegible] adrenaline in oil (1 mg daily) and of 1–[illegible] water as well as the separate effects o[illegible] of adrenaline in oil alone (1 mg every 2nd day), were studied for three months in 105 normal unsensitised albino rats A control group got no treatment The blood pressure was measured without anaesthesia The

average initial blood pressure before treatment was in each groups 110–114 mm Hg. In the adrenaline + sodium chloride groups there was found a significantly higher blood pressure after the 20th day of treatment than in the control and the sodium chloride groups. The highest blood pressure level during the whole test period was observed in the adrenaline + sodium chloride group. The difference in blood pressures between the adrenaline + sodium chloride group and the sodium chloride group was significant for almost the whole test period, but the difference between the adrenaline + sodium chloride group and the adrenaline group was significant only on the 18th–20th days of the test ($P < 0.01$). After treatment, the blood pressure tended to decrease in all groups. Our findings suggest a role for the combined effect of adrenaline and sodium chloride in the aetiology and development of hypertension.

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Pickering [illegible]

NFN name	Other Names
Acidum trethocanicum (pINN)	2 Hydroxy 2 6 10-trimethylundecanecarboxylic acid (1) (Against hypercholesterolaemia)
Alimemazinum tartrate alimemazini tartras (DCF pINN)	10-(3 Dimethylamino 2 methylpropyl)phenothiazine BAN Trimeprazine ® Theralene Vallergan (Psycho sedative)
Allylprodinum (BAN pINN)	3 Allyl 1 methyl-4 phenyl-4 propionoyloxypiperidine (Analgesic euforising)
Amanozinum (pINN)	2 Amino-4-anilino s triazine (Diuretic)
Ambazonum (BAN pINN)	1 Amidinohydrazono-4 thiosemicarbazonocyclohexa diene (2 5) ® Iversal (Antibacterial)
Aminopromazinum fumarate aminopromazini fumaras (DCF pINN)	10-(2 3 Bis(dimethylamino)propyl)phenothiazine BAN Proquamezine ® Lispamol (Anticonvulsant)
Amocainum chloride amocaini chloridum	3 (2 Diethylaminoethyl)-2 oxo 3 phenyl 2 3 dihydrogen benzofurane pINN Amolanonum (NND) ® Amethone hydrochloride (Local anaesthetic)
Amopyrochinum (pINN)	7 Chloro-4-(4 hydroxy 3-(pyrrolidinyl (1) methyl) anilino)quinoline pINN Amopyroquinum ® Propoquin (Chemotherapeutic)
Amphenidonum (pINN)	1-(3 Aminophenyl) 2 oxo 1 2-dihydrogenpyridine 1 (3 Aminophenyl) 2[1H]pyridone ® Dornwal (Psycho sedative)
Amydricainum chloride amydricaini chloridum nitrate amydricaini nitras (NNR 1946)	Benzoic acid (1 dimethylamino 2 (dimethylamino methyl) butyl (2)) ester ® Alypin hydrochloride (Local anaesthetic)
A oranatrium	~
	(Dye for determination of blood volume)
Beclamidum (BAN pINN)	N Benzyl 3-chloropropionamide ® Nydran (Anticonvulsant)
Benethidinum (BAN pINN)	1 (2 Benzyloxyethyl)-4-phenylpiperidine carboxylic acid-(4) ethyl ester (Analgesic euforising)
Benmalecenum (pINN)	N (2 3 Bis(4-chlorophenyl)-1 methylpropyl)maleic acid monoamide (one of the stereoisomers) (Uricosuric)

Acta pharmacol et toxicol 1961, 18, 218–230

Names approved by The Nordic Pharmacopoeia Council[1]) (NFN Names).

(Received March 4, 1961)

The Nordic Pharmacopoeia Council hereby announces that the Council has agreed upon an NFN[2]) name for each of the drugs mentioned in the list below This name may possibly be used if the drugs are included in the forthcoming Nordic Pharmacopoeia or if they are included in one or several of the National Nordic Pharmacopoeias or official formularies or addenda thereto

Information as to the drug that the NFN name covers is given as the chemical name of the drug, a selection of the registered trade names in common use and other names for pharmaceutical specialities (denoted ®) are given, together with the commonest non-registered names, which include the proposed or recommended International Non-Proprietary Names (denoted pINN and rINN) approved by WHO The registered trade name is in some instances the name of one of the salts mentioned or a preparation in which the drug in question is the active principle

BAN British Approved Name
DCF Denominations communes (françaises)
NND New and Nonofficial Drugs

Names approved in 1960[3]).

NFN name	*Other Names*
Acetyldigitoxinum (pINN,NND)	14 Acetyloxy 3β (O β D digitoxopyranosyl (1→4) O β D digitoxopyranosyl (1→4) β D digitoxopyranosyloxy) 5β card 20(22) enolide α Acetyldigitoxin ® *Acylanid* (Digitalislike)
Acetyloleandomycinum	Triacetic acid ester of oleandomycin pINN Triacetyloleandomycinum (NF) ® Cyclamycin Evramycin Matromycin Tao (Antibiotic)

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...isk Farmakopé nævn) for the Nordic

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NFN name	*Other Names*
Chlorthiazidum sodiumsalt chlorthiazidna trium (BP,DCF,pINN,USP)	6 Chloro-7 sulfamoyl 1,4,2 benzothiadiazinedioxide (1,1) pINN Chlorothiazidum (BP, DCF, USP) ® Chlotride, Diuresal Diuril, Diutrid, Ehydrid, Fenuril, Kloritsid, Lyovac diuril, Salisan, Saluric, Salutrid, Urinex. (Diuretic)
Chlorzoxazonum (BAN pINN,NND)	5 Chloro-2 oxo 2,3 dihydrogenbenzoxazole ® Neoflex, Paraflex (Skeletal muscle relaxant)
Cholini theophyllinas (BPC, pINN)	Salt of (2 hydroxyethyl)trimethylammonium hydroxide and 1,3 dimethyl 2 6 dioxo-1,2 3,6-tetrahydrogenpurine NND Oxtriphylline ® Choledyl Chophyllin, Kofyllin, Teokolin (Diuretic, vasodilator etc)
Clemizolum chloride clemizoli chloridum (BAN pINN)	1 (4 Chlorobenzyl)-2 (pyrrolidinyl (1) methyl)-benzimidazole ® Allercur (Antihistaminic)
Congo-ronnatrium	Disodium salt of 4 4 bis(1 amino-4-sulfonaphthyl (2)-azo)biphenyl Congo Red BPC Rubrum Congoensis (Dye for diagnostic use)
Cyclandelatum (BAN pINN)	Phenylglycolic acid (3,5 5 trimethylcyclohexyl) ester 3,5 5 Trimethylcyclohexylmandelate ® Cyclomandol Cyclospasmol (Spasmolytic vasodilator, periphere)
Cyclobenzaprinum (pINN)	5 (3 Dimethylaminopropylidene)dibenzo[a e]cycloheptatriene (Psycho-sedative)
Cyclopregnolum (BAN pINN)	6β-Hydroxy 3,5-cyclopregnanone-(20) ® Neurosteron (Psychopharmakon)
Demethylchlortetracyclinum chloride demethylchlortetracyclini chloridum (BAN pINN)	7 Chloro-4 dimethylamino 3 6 10,12 12a pentahydroxy
Dequalonum chloride dequaloni chloridum	1 1 Decamethylene (1,10)-bis(4 amino-2 methylquinolinium hydroxide pINN Dequalinii chloridum (BPC) ® Dekadin Dequadin (Antibacterial)
Dexbrompheniraminum maleate dexbrompheniramini maleas (pINN NND)	(+)-3 (4 Bromophenyl)-N,N-dimethyl 3 pyridyl (2)-propylamine ® Disomer (Antihistaminic)
Dexchlorpheniraminum maleate dexchlorpheniramini maleas	(+)-3 (4-Chlorophenyl)-N N-dimethyl 3 pyridyl (2)-propylamine ® Polaramine (Antihistaminic)

NFN name	*Other Names*
Bialamicolum chloride bialamicoli chloridum (BAN pINN)	3 3 Diallyl 5 5 bis(diethylaminomethyl) 4 4 dihydroxydiphenyl ® Camoform (Chemotherapeutic)
Bretylum 4 toluenesulfonate bretyli tosylas (BAN pINN)	N (2 Bromobenzyl) N-ethyldimethylammonium hydroxide pINN Bretylii tosylas ® Darenthin (Sympatholytic)
Brompheniraminum maleate brompheniramini maleas (pINN NND)	(±) 3 (4 Bromophenyl) N N dimethyl 3 pyridyl (2) propylamine ® Dimetane Ilvin (Antihistaminic)
Broparoestrolum (DCF pINN)	1 Bromo 2 (4 ethylphenyl) 1 2 diphenylethene ® Longestrol (Estrogen)
Butopyrammonii hydroxidum iodide butopyrammonii jodidum (pINN)	Butyldimethyl-(2 3-dimethyl 5 oxo 1 phenylpyrazol nyl (4))ammonium hydroxide (Analgesic antirheumatic)
Ceramacrogolum	Polyoxyethylene ethers of bees wax ® Atlas G 1726 (Pharmaceutical necessity)
Chaulmosulfonum (DCF pINN)	4 4 Bis(12 cyclopentyldodecanylcarboxamido (1))di phenylsulfone 4 4 B s(dihydrogenchaulmogram do) diphenylsulfone ® Chaulfone (Chemotherapeutic)
Cl loramanozu um	2 Amino 4 (4 chloran lino) s triazine pINN Chlorazanilum ® Orpidan (Diuretic)
Cl lorn e anonum (pINN)	2 (4 Chlorophenyl) 3 methyl 4 oxotetrahydrogen 1 3 4 thiazinedioxide (1 1) (Skeletal muscle relaxant)
Chlorphenesi ium (BAN pINN)	3 (4 Chlorophenoxy)propanediol (1 2) (Fungicide)
Cl lorphenoctonum salt with trans 1 2 bis(4 am no 2 sulfophenyl)ethene chlor phenoctoni amsonas	(2 4 Dichlorophenoxymethyl)d methyloctylammonium hydroxide pINN Chlorphenocti amsonas (BAN) (Antibacterial fungicide)
Chlorproguanilum chloride chlorproguanili chloridum (BAN pINN)	1 (3 4 Dichlorophenyl) 5 isopropylbiguanide ® Lapudrin (Chemotherapeutic)
Cl lorpropamidum (BAN pINN NND)	3 (4 Chlorobenzenesulfonyl) 1 propylcarbamide ® Diabaryl Diabet Diabines Diabinese Mellinese (Antid abetic)
Chlorprothixen im chloride chlorprothixeni chloridum (BAN pINN)	trans 2 Chloro 9 (3 dimethylaminopropylidene) thioxanthene ® Taractan Truxal (Psycho sedative)

NFN name	*Other Names*
Chlorthiazidum sodiumsalt chlorthiazidna trium (BP,DCF pINN,USP)	6 Chloro-7 sulfamoyl 1 4,2 benzothiadiazinedioxide-(1,1) pINN Chlorothiazidum (BP, DCF, USP) ® Chlotride, Diuresal, Diuril, Diutrid, Ehydrid, Fenuril, Kloritsid, Lyovac diuril, Salisan, Saluric, Salutrid, Urinex. (Diuretic)
Chlorzoxazonum (BAN pINN NND)	5 Chloro 2 oxo 2,3 dihydrogenbenzoxazole ® Neoflex Paraflex. (Skeletal muscle relaxant)
Cholini theophyllinas (BPC pINN)	Salt of (2 hydroxyethyl)trimethylammonium hydroxide and 1,3-dimethyl 2 6 dioxo-1,2,3,6-tetrahydrogenpurine NND Oxtriphylline ® Choledyl, Chophyllin, Kofyllin, Teokolin (Diuretic, vasodilator etc)
Clemizolum chloride clemizoli chloridum (BAN pINN)	1 (4 Chlorobenzyl) 2 (pyrrolidinyl (1)-methyl)-benzimidazole ® Allercur (Antihistaminic)
Congo onnatrium	Disodium salt of 4 4 bis(1 amino-4 sulfonaphthyl (2)-azo)biphenyl Congo Red BPC Rubrum Congoensis (Dye for diagnostic use)
Cyclandelatum (BAN pINN)	Phenylglycolic acid (3,5,5 trimethylcyclohexyl) ester 3 5 5 Trimethylcyclohexylmandelate ® Cyclomandol, Cyclospasmol (Spasmolytic, vasodilator, periphere)
Cyclobenzaprinum (pINN)	5 (3 Dimethylaminopropylidene)dibenzo(a e)cyclohepta triene (Psycho-sedative)
Cyclopregnolum (BAN pINN)	6β-Hydroxy 3,5-cyclopregnanone (20) ® Neurosteron (Psychopharmakon)
Demethylchlortetracyclinum chloride demethylchlortetracyclini chloridum (BAN pINN)	7 Chloro-4 dimethylamino-3 6 10,12 12a pentahydroxy-1 11-dioxo-1,4 4a 5,5a 6 11,12a octahydrogennaphthacenecarboxamide (2) ® Declomycin Ledermycin (Antibiotic)
Dequalonum chloride dequaloni chloridum	1 1 Decamethylene (1 10)-bis(4 amino-2 methylquinolinium hydroxide pINN Dequalinii chloridum (BPC) ® Dekadin, Dequadin (Antibacterial)
Dexbrompheniraminum maleate dexbrompheniramini maleas (pINN NND)	(+)-3-(4-Bromophenyl)-N N dimethyl 3 pyridyl (2)-propylamine ® Disomer (Antihistaminic)
Dexchlorpheniraminum maleate dexchlorpheniramini maleas	(+)-3 (4-Chlorophenyl)-N,N-dimethyl 3 pyridyl-(2)-propylamine ® Polaramine (Antihistaminic)

NFN-name	*Other Names*
Diampromidum (pINN)	N-(2-(N-Methylphenethylamino)propyl)-N-phenylpropionamide. (Analgesic)
Diathymosulfonum (DCF, pINN)	4,4'-Bis(4 hydroxy-2-methyl-5 isopropylphenylazo)-diphenylsulfone Ⓕ Diatox argentique (silver-compound) (Chemotherapeutic)
Dichlormezanonum (pINN)	2-(3,4 Dichlorophenyl)-3 methyl-4-oxotetrahydrogen-1 3,4 thiazinedioxide-(1,1) (Psycho sedative)
Dichlorphenarsinum chloride dichlorphenarsini chloridum (BAN,DCF,rINN,Ph Int)	3-Amino-4 hydroxyphenyldichloroarsine. Previous NFN name Chlorphenarsinum rINN, Ph Int Dichlorophenarsini hydrochloridum (BAN, DCF) Ⓓ Clorarsen, Dichlor-mapharsen (Chemotherapeutic)
Dihydrocodeinum (pINN)	6-Hydroxy-3 methoxy-17-methyl-4,5-epoxymorphinane. Dihydrocodeine (Analgesic, euforisine)
Diloxanidum (BAN pINN)	4 Hydroxy-(N-methyldichloroacetamido) benzene. Ⓕ Entamide (Chemotherapeutic)
Dimeth[illegible]rum (pINN)	7 (2 Dimethylaminoethyl)-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydrogenpurine. 7-(2 Dimethylaminoethyl)-1,3-dimethylxanthine (Psychoanaleptic)
Dimethisteronum (BAN,pINN)	6α-Methyl 17α methylethynyl-17β-hydroxy-3-oxoandrostene-(4). 6α, 21-Dimethylethisterone Ⓑ Secrosteron (Progestogen)
Diphenoxylatum (BAN pINN)	1-(3 Cyano-3,3-diphenylpropyl)-4 phenylpiperidine-carboxylic acid-(4) ethyl ester (Analgesic, euforisine)
Diphoxazidum (pINN)	N Acetyl-N'-(3 hydroxy-3,3-diphenylpropionyl)-hydrazine (Psychosedative)
Dithiazaninum iodide dithiazanini iodidum (BAN pINN NND)	3 Ethyl-2-(5-(3-ethylbenzothiazolinylidene-(2))-pentadiene-(1,3)-yl)benzothiazolium hydroxide. Ⓓ Abminthic, Delvex, Helmsum, Netocyd, Telmicid, Telmid (Anthelmintic)
Enallylpropymalum sodium methohexital natrium	(±)-5-Allyl-1-methyl-5-(1-methylpentyn-(2)-yl)-2,4,6-trioxotetrahydrogenpyrimidine (±)-5-Allyl-1-methyl-5-(1-methylpentynyl-(2))barbituric acid. pINN Methohexitalum Ⓓ Brietal natrium, Methohexital (Anaesthetic)

NFN name	Other Names
Fibrinolysinum humanum (pINN)	Enzyme produced from human plasma by treating pro fibrinolysine with streptokinase Plasmin pINN Fibrinolysinum (humanum) ® Actase Fibrinolysin (human) lyovac Thrombolysin lyovac (Enzyme)
Flumethiazidum (BAN pINN)	7 Sulfamoyl 6-trifluoromethyl-4H 1 2 4-benzo thiadiazinedioxide (1 1) ® Ademol (Diuretic)
Fluormetholonum (pINN NND)	9α Fluoro-11β 17α dihydroxy 6α methyl 3 20-dioxopregnadiene (1,4) pINN Fluorometholonum (NND) ® Oxylone (Glucocorticoid)
Fluormethylprednisolonum acetic acid ester fluormethylprednisoloni acetas	9α Fluoro-11β 17α,21 trihydroxy 16α methyl 3 20 dioxopregnadiene (1 4) 9α Fluoro-16α methylprednisolone pINN Dexamethasonum (BAN NND) ® Decadron Deltafluoren N Deronil Dexacortal Dexacortisyl Dexameson Flumeprednisolon Forte cortin Gammacorten Millicorten Oradexon (Glucocorticoid)
Fluoxiprednisolonum acetic acid di ester fluoxiprednisoloni acetas	9α Fluoro 11β 16α,17α 21 tetrahydroxy 3 20 dioxopregnadiene (1 4) 9α Fluoro 16α hydroxyprednisolone pINN Triamcinolonum (BAN NND) ® Aristocort Kenacort Ledercort (Glucocorticoid)
Furethidinum (BAN pINN)	4 Phenyl 1 (2 tetrahydrogenfurfuryloxyethyl) piperidinecarboxylic acid (4) ethyl ester (Analgesic euforising)
Glybuthiazolum	5 Tert butyl 2 sulfanilamido 1 3 4 thiadiazole pINN Glybuthiazolum (DCF) ® Glypasol (Antidiabetic)
Glyprothiazolum	5 Isopropyl 2 sulfanilamido-1 3 4-thiadiazole pINN Glyprothiazolum (DCF) (Antidiabetic)
Griseofulvinum (BAN pINN)	Antibiotic produced by Penicillium griseofulvum and Penicillium janczewskii 7 Chloro-2 4 6-trimethoxy 6 methyl 3 4 -dioxospiro[benzofurane 2(3H) 1 [2] cyclohexene] ® Fulcin Fulvicin Fungivin Grifulvin Grisovin Lamoryl. (Antibiotic)
Hedachinum chloride hedachini chloridum (BAN pINN)	2,2 Hexadecamethylene-(1 16)-bisisoquinolini umhydroxide) pINN Hedaquinii chloridum (BAN) ® Teoquil. (Fungicide)

NFN-name	*Other Names*
Diampromidum (pINN)	N-(2-(N-Methylphenethylamino)propyl)-N-phenylpropionamide (Analgesic)
Diathymosulfonum (DCF, pINN)	4,4'-Bis(4-hydroxy-2-methyl-5-isopropylphenylazo) diphenylsulfone ® Diatox argentique (silver-compound) (Chemotherapeutic)
Dichlormezanonum (pINN)	2-(3,4-Dichlorophenyl)-3-methyl-4-oxotetrahydrogen-1,3,4-thiazinedioxide-(1,1) (Psycho sedative)
Dichlorphenarsinum chloride· dichlorphenarsini chloridum (BAN,DCF,rINN,Ph Int)	3-Amino-4-hydroxyphenyldichloroarsine Previous NFN-name Chlorphenarsinum rINN, Ph Int Dichlorophenarsini hydrochloridum (BAN, DCF) ® Clorarsen, Dichlor-mapharsen (Chemotherapeutic)
Dihydrocodeinum (pINN)	6-Hydroxy-3-methoxy-17-methyl-4,5-epoxymorphinane Dihydrocodeine (Analgesic, euforising)
Diloxanidum (BAN,pINN)	4-Hydroxy-(N-methyldichloroacetamido) benzene ® Entamide (Chemotherapeutic)
Dimethazanum (pINN)	7-(2-Dimethylaminoethyl)-1,3-dimethyl 2,6-dioxo-1,2,3,6-tetrahydrogenpurine 7-(2-Dimethylaminoethyl)-1,3-dimethylxanthine (Psychoanaleptic)
Dimethisteronum (BAN,pINN)	6α-Methyl-17α-methylethynyl-17β-hydroxy-3-oxoandrostene-(4) 6α, 21-Dimethylethisterone ® Secrosteron (Progestogen)
Diphenoxylatum (BAN,pINN)	1-(3-Cyano-3,3 diphenylpropyl)-4-phenylpiperidine-carboxylic acid-(4) ethyl ester (Analgesic, euforising)
Diphoxazidum (pINN)	N-Acetyl N -(3-hydroxy 3,3 diphenylpropionyl) hydrazine (Psycho-sedative)
Dithiazaninum iodide dithiazanini jodidum (BAN,pINN,NND)	3-Ethyl-2-(5-(3 ethylbenzothiazolinylidene-(2))-pentadiene-(1,3) yl)benzothiazolium hydroxide ® Abminthic, Delvex, Helmisin, Netocyd, Telmicid, Telmid (Anthelmintic)
Enallynymalum sodiumsalt enallynymal-natrium	(±)-5-Allyl-1-methyl 5-(1 methylpentyn (2)-yl)-2,4,6-trioxotetrahydrogenpyrimidine (±)-5-Allyl-1-methyl 5 (1-methylpentyne (2)-yl)barbituric acid pINN Methohexitalum ® Brietal natrium, Methoxital (Anaesthetic)

NFN name	*Other Names*
Fibrinolysinum humanum (pINN)	Enzyme produced from human plasma by treating profibrinolysine with streptokinase Plasmin pINN Fibrinolysinum (humanum) ® Actase, Fibrinolysin (human) lyovac, Thrombolysin lyovac (Enzyme)
Flumethiazidum (BAN pINN)	7 Sulfamoyl 6 trifluoromethyl-4H 1,2,4 benzothiadiazinedioxide (1 1) ® Ademol (Diuretic)
Fluormetholonum (pINN NND)	9α Fluoro-11β, 17α dihydroxy 6α methyl 3 20-dioxopregnadiene (1 4) pINN Fluorometholonum (NND) ® Oxylone (Glucocorticoid)
Fluormethylprednisolonum acetic acid ester fluormethylprednisoloni acetas	9α Fluoro 11β 17α 21 trihydroxy-16α methyl 3,20-dioxopregnadiene (1,4) 9α Fluoro 16α methylprednisolone pINN [illegible] cortin, Gammacorten, Millicorten, Oradexon, (Glucocorticoid)
Fluoxiprednisolonum acetic acid diester fluoxiprednisoloni acetas	9α Fluoro 11β 16α,17α 21 tetrahydroxy 3,20 dioxopregnadiene (1 4) 9α Fluoro 16α hydroxyprednisolone pINN Triamcinolonum (BAN NND) ® Aristocort, Kenacort Ledercort *(Glucocorticoid)*
Furethidinum (BAN pINN)	4 Phenyl 1 (2 tetrahydrogenfurfuryloxyethyl)-piperidinecarboxylic acid (4) ethyl ester (Analeesic, euforising)
Glybuthi olum	5 Tert butyl 2 sulfanilamido 1,3,4 thiadiazole pINN Glybuthiazolum (DCF) ® Glypasol (Antidiabetic)
Glyprothi olum	5 Isopropyl 2 sulfanilamido 1,3,4 thiadiazole pINN Glyprothiazolum (DCF) (Antidiabetic)
Griseofulvinum (BAN pINN)	Antibiotic produced by Penicillium griseofulvium and Penicillium janczewskii 7 Chloro-2 ,4 6 trimethoxy 6 -methyl 3 4 dioxospiro[benzofurane-2(3H) 1 [2] cyclohexene] ® Fulcin Fulvicin Fungivin, Grifulvin, Grisovin Lamoryl (Antibiotic)
Hedachinum chloride hedachini chloridum [illegible]	2 2 Hexadecamethylene (1 16) bis(isoquinolinium hydroxide) 4 Hedaquinii chloridum (BAN) Teoquil gicide)

NFN name	*Other Names*
Hexadimethrinum bromide hexadimethrini bromidum (pINN)	N N N N Tetramethylhexamethylened amine N trimethylenedibromide polymer ® Polybrene Polyerone (Heparin antagonist)
Hydrochlorthiazidum (BAN pINN NND)	6 Chloro 7 sulfamoyl 3 4 d hydrogen 1 2 4 benzothiadiazinedioxide (1 1) pINN *Hydrochlorothiazidum* (BAN NND) ® Dichlotride Dihydran Direma Ehydrid novum Esidrex Esidrix Hydrex Hydril Hydrodiuril Hydro saluric Oretic (Diuretic)
Hydroxidioni natrii succinas (BAN pINN NND)	Sodiumsalt of 21 (3 carboxypropionyloxy)pregnad one (3 20) pINN Hydroxyd oni natrii succinas (BAN NND) ® Presuren Viadril (Anaesthetic)
Imipraminum chloride imipramini chloridum (BAN pINN)	5 (3 Dimethylaminopropyl) 10 11 dihydrogen 5H dibenz[b f]azepine ® Tofranil (Psychoanaleptic)
Inprochonum (BAN pINN)	2 5 Bisethyleneimino 1 4 dioxo 3 6 d propoxy cyclohexadiene (2 5) pINN Inproquonum (BAN) (Nucleotoxic agent)
Isoproponum iodide isoproponi jodidum	(3 Carbamoyl 3 3 diphenylpropyl)methyldi sopropyl ammonium hydroxide pINN Isopropamidi iodidum (BAN NND) ® Darbid Tyrimide (Ant cholinergic)
Isoxsuprinum (pINN)	1 (4 Hydroxyphenyl) 2 (1 methyl 2 phenoxyethyl amino)propanol (1) ® Duvadilan Vasodilan (Vasodilating)
Kanamycinum sulfate kanamycini sulfas (BAN pINN NND)	Antibiotic produced by Streptomyces kanamyceticus ® Kanacedin Kanacin Kanecidin Kannasyn Kantrex Kantrox (Antib otic)
Khellosidum (DCF pINN)	7 Hydroxymethyl 4 methoxy 5 oxo 5H furo[3 2 g] [1]benzopyrane 7 glucoside 2 Hydroxymethyl 5 methoxyfuranochromon glucoside ® Khellinin (Coronary vasodilating)
Levisoprenalinum (DCF pINN)	( ) 1 (3 4 Dihydroxyphenyl) 2 sopropylaminoethanol (Sympathomimetic)
Levophenacylmorphan im (BAN pINN)	( ) 3 Hydroxy 17 phenacylmorphinane (Analges c euforis ng)
Levorphanolum tartrate levorphanoli tartras (BPC rINN NND)	( ) 3 Hydroxy 17 methylmorphinane Previous NFN name Levorphanum ® Dromoran Levo dromoran tartrate (Analges c euforising)

NFN name	*Other Names*
Lysergidum (pINN)	7 Methyl-4 6 6a 7,8 9 hexahydrogenindolo[4 3 fg] quinol necarboxylic acid (9) d ethylamide Lysergic acid d ethylamide (Psychosomimetic)
Mannomustinum chloride mannomustini chloridum (BAN pINN)	1 6 B s(2 chloroethylamino)-1 6 dideoxy D mannitol ® Degranol (Nucleotoxic agent radiomimetic)
Melphalanum (BAN pINN)	3 (4 Bis(2-chloroethylamino)phenyl)-L alanine ® Alkeran (Nucleotoxic agent radiomimetic)
Metazocinum (BAN pINN)	8 Hydroxy 3 6 11 trimethyl 1 2,3 4 5 6-hexahydrogen 2 6 methano 3 benzazocine (Analgesic euforising)
Methocamphonum methylsulfate methocamphoni methylsulfas	(+) 3 (3 Trimethylammoniumpropyl 1 3 8 8 tetra methyl 3 azan umbicyclo[3 2 1]octane dihydroxide pINN Trimethid nii methosulfas (BAN) ® Camphidonium Ostensin Ostensol (Ganglionic blocking agent)
Methocarbamolum (BAN pINN NND)	Carbamic acid (2 hydroxy 3-(2 methyxyphenoxy) propyl ester ® Robaxin Tresortil (Skeletal muscle relaxant)
Methylprednisolonum acetic acid ester methylpredni soloni acetas (BAN pINN NND)	11β 17α 21 Trihydroxy 6α methyl 3 20 dioxo pregnadiene (1 4) 6-Methylprednisolone ® Depo medrol Medrelon Medrol Medrone Mesopren Urbason (Glucocorticoid)
Nitrofuradoxonum	5 Methyl 3 (5 nitrofurfuryl deneamino)oxazolidinone (2) pINN Furmethoxadonum (Chemotherapeutic)
Nitrofurmethonum	5 Morpholinomethyl 3 (5 nitrofurfurylideneamino) oxazol dinone-(2) pINN Furmethonolum ® Altafur (Chemotherapeutic)
N trofuroxizonum	1 (2 Hydroxyethyl) 1 (5 nitrofurfurylideneamino)-carbamide rINN Nidroxyzonum (Antibacterial)
Norlevorphanolum (BAN pINN)	( ) 3 Hydroxymorphinane (Ana[illegible]
Orphenad num chlor de orphenadin chloridum citrate orphenadini citras	[illegible] para[illegible] drug)
Oxadimed num (pINN)	N (Benzoxazolyl (2)) N benzyl N N dimethylethylene diamine

NFN name	*Other Names*
Oxanamidum (pINN,NND)	1 2 Epoxy 1 ethylpentanecarboxylic acid (1) amide 2 Ethyl 3 propylglycidamide ® Quiactin (Psycho sedative)
Oxibuprocainum chloride oxibuprocaini chloridum (DCF,pINN)	4 Amino 3 butoxybenzoic acid (2 diethylaminoethyl) ester pINN Oxybuprocainum (DCF) USP Benoxinate Hydrochloride ® Dorsacaine hydrochloride Novesin (Local anaesthetic)
Oxichlorochinum sulfate oxichlorochini sulfas	7 Chloro 4 [4 (N ethyl N (2 hydroxyethyl)amino) 1 methylbutylamino] quinoline pINN Hydroxychloroquinum (BP USP) ® Heliopar Plaquenil (Chemotherapeutic)
Oxindasolum	3 (2 Aminoethyl) 5 hydroxy 1 (4 methoxybenzyl) 2 methylindole pINN Hydroxindasolum (Enteramin antagonist)
Oxiphenbutazonum (pINN)	4 Butyl 1 (4 hydroxyphenyl) 2 phenylpyrazolidine dione (3 5) pINN Oxyphenbutazonum (Antirheumatic)
Oxiphenisatinum (pINN)	3 3 Bis(4 hydroxyphenyl) 2 oxo 2 3 dihydrogenindole pINN Oxyphenisatinum ® Lavema (Laxative)
Oxiprogesteronum caproic acid ester oxiprogesteroni caproas acetic acid ester oxiprogesteroni acetas	17α Hydroxy 3 20 dioxopregnene (4) 17α Hydroxyprogesterone pINN Hydroxyprogesteroni acetas (NND) ® Delalutin Primolut depot Prodox Proluton depot (Progestogen)
Pantothenolum	(+) 3 (2 4 Dihydroxy 3 3 dimethylbutaneamido) propanol (1) Pantenol ® Bepanthen Pantevit Panthoderm Pantol (Vitamin (B complex))
Paracetamolum (BAN pINN)	1 Acetamido 4 hydroxybenzene NF Acetaminophen ® Alvedon Febrilix Napatyl Panadol Tylenol (Analgesic antipyretic)
Paridocainum (pINN)	4 Butylaminobenzoic acid (1 methylpiperidyl (4)) ester (Local anaesthetic)
Pasiniazidum (DCF pINN)	Salt of 4 amino 2 hydroxybenzoic acid and pyridyl (4) carbonylhydrazine Isonicotinoylhydrazine 4 amino salicylate (Chemotherapeutic)
Paucimycinum	Antibiotic produced by Streptomyces paucisporogenes pINN Hydroxymycinum

NFN name	Other Names
Pecazinum acetate pecazini acetas chloride pecazini chloridum (BAN pINN)	10-(1 Methylpiperidyl (3) methyl)phenothiazine NND Mepazine ® Lacumin Pacatal (Psycho sedative)
Pempidinum bitartrate pempidini bitartras (BAN pINN)	1 2 2 6 6-Pentamethylpiperidine ® Perolysen Tenormal (Ganglionic blocking agent)
Penicillinasum (pINN NND)	Penicillin hydrolysing enzyme ® Neutrapen (Enzyme)
Penicillinclemizolum	Salt of 4-carboxy 5,5 dimethyl 2 (1 (phenylacetamido) carboxymethyl)thiazolidine β-lactam and 1 (4-chloro-benzyl) 2-(pyrrolidinyl (1) methyl)benzimidazole Benzylpenicillin salt of clemizol pINN Clemizolum penicillinum (BAN) ® Lergopenin Megacillin Neopenyl (Antibiotic)
Penicillinphenyrazinum	D salt of 4-carboxy 5 5-dimethyl 2 (1 (phenylacetamido) carboxymethyl) thiazolidine β-lactam and 2,5 diphenyl p perazine Di(benzylpenicillin)salt of 2 5-diphenylpiperazine pINN Phenyracillinum (DCF) ® Alfadryl (Antibiotic)
Perphenazinum (BAN pINN NND)	2 Chloro 10 [3 (1 (2 hydroxyethyl)piperazinyl(4)) propyl]phenoth azine ® Fentazin Trilafon (Psycho-sedative)
Phenactropinum chloride phenactropini chloridum (BAN pINN)	Hydroxide of (±)-8 phenacyl 3 phenylglycoloyl oxytropane N Phenacylhomatropine pINN Phenactropinii chloridum (BAN) ® Trophenium (Hypotensive)
Phenelzinum sulfate phenelzini sulfas (BAN pINN)	Phenethylhydrazine ® Monofen Nardil (Psychoanaleptic)
Phenethanolum	2 Phenylethanol NF Phenylethyl alcohol (Pharmaceutical necessity)
Phenethylazocinum bromide phenethylazocini bromidum	8 Hydroxy 6 11 dimethyl 3 phenethyl 1 2 3 4 5 6 hexahydrogen 2 6-methano 3 benzazocine pINN Phenazocinum (BAN) ® Narphen Prinadol (Analgesic euforising)
Pheniraminum maleate pheniramini maleas (BAN pINN NF)	N N Dimethyl 3 phenyl 3 pyridyl (2)-propylamine Prophenpyridamine ® Trimeton (Antihistaminic)

NFN-name	*Other Names*
Phenmetralinum chloride phenmetralini chloridum	(±)-3-Methyl-2-phenylmorpholine pINN: Phenmetrazinum (BPC,NND) DCF: Oxazimédrine ®. Minadit, Preludin (Anorexigene)
Phosphothiaminum	3-((4-Amino-2-methylpyrimidinyl (5))methyl)-4 methyl-5-(2-phosphonooxyethyl)thiazolium chloride Thiamin-monofosforic acid ester pINN Monophosphothiaminum (DCF) *(Vitamin)*
Piminodinum (BAN,pINN)	1-(3-Anilinopropyl)-4-phenylpiperidinecarboxylic acid-(4) ethylester. (Analgesic, euforising)
Poloxalkolum (pINN,NND)	Ethyleneoxide-propyleneoxide-propyleneglycol polymer ®· Polykol (Bulk laxative)
Polybenzarsolum (pINN)	3-Arsono 6-hydroxyphenyl-(1)-methylene-polymer. (Chemotherapeutic)
Poskinum bromide poskini bromidum (BAN,pINN)	3-(2-Phenyl-2-(propionoyloxymethyl)acetyloxy) 6,7-epoxytropane Propionoylhyoscine ® Proscopine (Tropane-alkaloid)
Pralidoximum iodide pralidoximi jodidum (BAN,pINN)	1-Methyl-2-hydroxyiminomethylpyridinium hydroxide 2-Pyridinealdoxime methoiodide (the iodide) PAM pINN Pralidoximi methiodidum ® Protopam (Anticholinesterase antagonist)
Propethonum iodide propethoni jodidum chloride propethoni chloridum	Triethyl(3-cyclohexyl-3-hydroxy-3-phenylpropyl)-ammonium hydroxide pINN Tridihexethyli iodidum (NND) ® Pathilon, Pathilon N (Anticholinergic)
Protamini sulfas (pINN,NF)	Protamine sulfate *(Protein, used as heparin antagonist)*
Proxiphyllinum (BAN,pINN)	7-(2-Hydroxypropyl)-1,3-dimethyl 2,6-dioxo-1,2,3,6-tetrahydrogenpurine 7-(2-Hydroxypropyl)theophylline pINN Proxyphyllinum (BAN) ® Brontyl, Neofyllin, Purophyllin, Theoden, Theon *(Diuretic, vasodilator etc)*
Pyrazinamidum (BAN,pINN,NND)	Pyrazinecarbocylic acid (2) amide ® Aldenamide, Isopyratsin, Tisamid (Chemotherapeutic)
Renanolonum (pINN)	3α-Hydroxypregnadione-(11,20) (Anaesthetic)
Ricinomacrogolum	Polyoxyethylene ethers of ricinolic acid ® Atlas G 1295 (Pharmaceutical necessity)
Ristocetinum (BAN,pINN,NND)	Mixture of antibiotics produced by Nocardia lurida ® *Spontin*

NFN name	*Other Names*
Salinazidum	N-(Pyridyl-(4)-carbonyl)-N' salicylidenehydrazine. N Isonicotinoyl N' salicylidenehydrazine pINN Salinazidum (BAN) ® Acozid Nicozid, Nupasal (Chemotherapeutic)
Sulfachlorpyridazinum (pINN)	6-Chloro 3 sulfanilamidopyridazine ® Consulid (Chemotherapeutic)
Sulfadimethoxinum (BAN pINN NND)	2 4-Dimethoxy 6-sulfanilamidopyrimidine BAN Sulphadimethoxine ® Madribon Madriqid (Chemotherapeutic)
Sulfaethidolum (BAN pINN NND)	5 Ethyl 2 sulfanilamido 1 3 4 thiadiazol BAN Sulphaethidol DCF Sulfaéthylthiodiazol. ® Sethadil, Sulfa perlongit Sul spansion, Sul spantab (Chemotherapeutic)
Sulfamethoxipyridazinum (BAN pINN USP)	6-Methoxy 3 sulfanilamidopyridazine pINN Sulfamethoxypyridazinum (NND) BAN Sulphamethoxypyridazine DCF Sulfaméthopyrazine ® Durasulf, Kynex, Lederkyn, Longamid Longin, Longisulf Midicel Midikel Nidikel, Sulfametopyridazin Sulfdurazin Volocid (Chemotherapeutic)
Sulfinpyrazonum (BAN pINN)	1,2 Diphenyl-4-(2 phenylsulfinylethyl)pyrazolidindione (3 5) BAN Sulphinpyrazone ® Anturan Anturanil (Uricosuric)
Sulfobromphthaleinum d sod umsalt sulfobromphthaleinnatrium	3 3 Bis(4 hydroxy 3 sulfophenyl)-4 5 6 7 tetrabromo 1-oxophthalane Tetrabromophenolphthaleindisulfonic acid USP Sulfobromophthalein Sodium ® Bromsulphalein Sodium (Agent for liver function test)
Sulocarbilatum (pINN)	4 Sulfamoylphenylcarbamic acid (2 hydroxyethyl) ester (Carboanhydrase inhibitor)
Syrosingopinum (pINN NND)	2 11 Dimethoxy 1 methoxycarbonyl 3 (4 ethoxycarbonyloxy 3 5 dimethoxybenzoyloxy) 1,2 3 4 4a 5 7 8 13, 13b 14 14a dodecahydrogenbenz[g] ndolo[2 3 a]quinolizine 4 Ethoxycarbonyl 3 5 dimethoxybenzoic acid ester of methylreserpate Carbethoxysyringoyl methyl reserpate ® Singoserp (Psycho-sedative)
Tacrinum (BAN pINN)	9 Amino-1 2 3 4 tetrahydrogenacridine (Curare antagonist)
Tetrahydrozolinum chloride tetrahydrozolini chloridum (BAN NF)	2 (1 2 3 4 Tetrahydrogennaphthyl (1))imidazoline rINN Tetryzolinum ® Tyzanol Tyzine hydrochloride (Sympathomimetic)

NFN name	*Other Names*
Thalidomidum (BAN pINN)	2 6 Dioxo 3 phthalimidopiperidine N Phthalylglutamic acid imide ® Distaval Lulamin Neosedyn Neurosedyn (Sedative)
Thialisobumalnatrium	Mixture of sodiumsalt of 5 allyl 5 isobutyl-4 6 dioxo-2 thiohexahydropyrimidine and 6 % dryed sodium carbonate Sodium 5 allyl 5 isobutyl 2 thiobarbiturate and 6 % dryed sodium carbonate pINN Buthalitalum natricum BAN Buthalitone Sodium ® Baytinal Transithal Ulbreval (Anaesthetic)
Thiambutosinum (BAN pINN)	1 (4 Butoxyphenyl) 3 (4 dimethylaminophenyl) thiocarbamide (Chemotherapeutic)
Thiocolchicosidum (DCF pINN)	7 Acetamido 3 glucopyranosyloxy 1 2 dimethoxy 10 methylthio 9 oxo 5 6 7 9 tetrahydrogenbenzo[a]heptalene 2 14 Di(demethoxy) 2 glucosidoxy 14 methyl thiocolchicine ® Coltramyl (Derivative of colchicin)
Thiopropazatum chloride thiopropazate chloridum (BAN pINN NND)	10 [3(4 (2 Acetyloxyethyl)piperazinyl (1))propyl] 2 chlorophenothiazine ® Dartal dihydrochloride Dartalan (Psycho sedative)
Thioridazinum chloride thioridazini chloridum (BAN pINN)	10 [2 (1 Methylpiperidyl (2))ethyl] 2 methylthiophenothiazine ® Malloryl Mellaril Melleril (Psycho sedative)
Triacetinum (pINN NND)	Propanetriol (1 2 3) triacetic acid ester Glyceroltriacetic acid ester ® Enzacetin Fungacetin (Fungicide pharmaceutical necessity)
Tropiglinum (BAN pINN)	3 (2 Methylcrotonoyloxy)tropane Tiglyltropine (Antiparkinson agent)
Xylometazolinum chloride xylometazolini chloridum (pINN)	2 ((4 *Tert* butyl 2 6 dimethylphenyl)methyl) 2 imidazoline ® Otrivin *(Vasoconstrictor)*

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From the University Institute of Forensic Medicine, Copenhagen

The Urinary Excretion of Ethanol by Man

By

Frank Lundquist

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Knowledge of the laws governing the excretion of alcohol through the kidneys is important for the forensic evaluation of analytical findings when urine has been examined in connection with accidents or violation of traffic rules The use of urine analysis as important or sole evidence in such circumstances has been advocated by several authors (HAGGARD, GREENBERG, CARROLL & MILLER 1940, SOUTHGATE & CARTER 1926, cf ELLERBROOK & VAN GAASBEEK 1943) Also, when blood analyses are available the ethanol concentration in a urine sample voided at the time of taking the blood sample may give important information about the case in particular, it should be possible to calculate a lower limit for the maximum blood ethanol concentration in the period during which the urine has been produced by the kidneys For such purposes it is clearly necessary to know the degree of confidence with which the blood alcohol concentration may be calculated from the concentration in the urine

It is generally assumed (HAGGARD & GREENBERG 1934) that the ethanol concentration of urine leaving the kidney is nearly the same as that of the water phase of the blood perfusing the organ A capacity of the kidney to concentrate alcohol has never been demonstrated The published material however, is too limited to permit the conclusion that the human kidney can never produce urine with a concentration of ethanol higher than that of the water phase of the blood at the same time The most conclusive kind of experiments for elucidating this problem would be to collect urethral urine continuously during infusion of ethanol at a rate sufficient to keep the blood concentration nearly constant, while the urine flow is varied within wide limits Such experiments have not been performed and for practical reasons could not be on a sufficiently large scale

As alternatives to this approach two procedures have been used in the work reported here The first consists simply in collecting urine as

frequently as possible during an experiment *in which the* blood ethanol concentration is measured at intervals sufficiently close to permit a reliable average blood ethanol concentration to be computed for each of the periods of urine collection The other procedure is a statistical one, based on a number of analyses conducted for the police

Methods.

In most experiments blood samples were taken from the earlobe in Widmark tubes and analysed by the method of WIDMARK (1932) (see LUNDQUIST 1959) Urine was analysed in the same way Blood analyses were usually done in triplicate and urine analyses in duplicate In two experiments blood was taken from the cubital vein and serum used for the analyses for these the enzymic method for ethanol determination, in LUNDQUIST'S (1959) modification, was used Creatinine was determined by the Jaffe reaction

The experimental subjects were all males aged 22–55 years They were given about 1 g of ethanol/kg body weight as red wine or brandy In most experiments the alcohol was given in the course of one hour, and the blood sampling was begun about one hour after all the ethanol had been consumed In a few experiments the alcohol was given in two periods at an interval of two hours

The blood (or serum) ethanol concentration was calculated as the average for each urine collection period from the blood alcohol curve This was obtained by connecting the experimental blood ethanol values by straight lines No attempt was made to smooth out the experimental curve

Results.

In all, 19 experiments were performed on 16 experimental subjects There were 61 urine collection periods, from which a reasonably certain average value for the blood ethanol concentration was obtained The possible sources of inaccuracy in these experiments are mainly three

1 The presence of residual urine in the vesica will result in mixture of urine with that formed during the preceeding collecting period This does not seem to cause serious errors, as the ratio of urine to blood alcohol concentration (designated below U/B ratio) is not significantly different at increasing and decreasing blood alcohol concentrations, as seen from table 1

2 The rate of urine flow changes during the collection period This results in uncertainty about the average blood ethanol concentration to be employed, as this has generally not been constant during the period, and may undoubtedly sometimes cause serious error, especially when the period is long and the blood ethanol concentration low At high blood ethanol concentrations errors from this source will be insignificant

Table 1.

The ratio urine ethanol concentration/blood ethanol concentration
17 experiments involving 15 male subjects
The blood ethanol concentration was in the range 0 5–1 5 g/l

Change of blood ethanol concentration during the urine collection periods	Number of periods	Average urine blood ethanol conc	Range
Rising	12	1 34	1 16–1 51
Nearly constant	8	1 36	1 26–1 47
Falling	5	1 35	1 32–1.51
Total	55	1 35	

In only three of the experiments reported here were the urine flow both in the preceeding and the following period nearly the same The U/B ratio then were 1 26, 1 23, and 1 30 In most of the collection periods both urine flow and blood ethanol concentration were decreasing This means that *the urine/blood ratios will show a tendency towards too high values*

3 The analytical error of the ethanol determination in blood and urine may cause appreciable spreading of the U/B ratio If we take as an example a period in which the blood ethanol concentration is 0 8 g/l and the corresponding urine concentration 1 04 g/l, an error of 0 05 g/l, which is quite common for the analytical methods employed, will give as extreme values for the U/B ratio 1 16 and 1 45 (i e, when the errors are in opposite directions) It is therefore evident that the range of values found in these experiments is compatible with the assumption of a constant ratio of the U/B ratio

Table 2 shows that the U/B ratio does not change significantly with the rate of urine production This is also evident from table 3 where it is seen that urine flow as low as 0 2 ml/min gave the same urine/serum ratio as was found with a urine flow of 3 6 ml/min

The 10 periods showing the highest U/B ratios (1 47–1 51) come from 8 different experiments In 6 of these periods the relative accuracy with

Table 2

Influence of the urine flow on the ratio urine ethanol concentration blood ethanol concentration The experiments are the same as those used for Table 1

Urine flow (ml/min)	Number of periods	U/B ratio	Range
<1	10	1 31	1 13–1 47
1–2	9	1 32	1 12–1 51
2–16	36	1 37	1 16 1 51

Table 3.

The ratio of urine to blood serum ethanol concentrations Two experiments on one experimental subject (age 22 years)

Serum ethanol concentration (g/l)	Length of period (min)	Urine flow ml/min	Urine/serum ratio
0 85	37	3 6	1 11
0 73	39	0 69	1 08
0 56	41	0 44	1 09
0 45	41	0 2	1 11
0 79	50	2 3	1 08
0 66	37	0 9	1 05
		Average	1 07

which the blood ethanol concentration was estimated was less than normal, either because the level of ethanol was low or because the individual values showed abnormal spread In two periods the blood ethanol concentration was rising rapidly, which might easily have introduced errors in the determination of the average value for the periods in question It may therefore be justifiable to omit these 9 collection periods from the material, and then the average U/B ratio decreases to 1 32

Forensic Cases submitted for Analysis

It is sometimes possible to draw conclusions about the U/B ratio from blood and urine samples taken at the same time If the amount of urine voided is not too large, and consequently the period during *which it has* been produced relatively short, and if at the same time the blood-ethanol concentration is high, the U/B ratio should deviate little from the one valid at the time of urine production

The question of the rate of urine production was studied by measuring the concentration of creatinine in the urine As seen from fig 1 the creatinine concentration is much lower (the rate of urine production therefore higher) in persons with a high blood ethanol concentration than in persons whose blood is devoid of ethanol Less than 1% of the group with more than 0 75 g/l of ethanol in the blood have creatinine values above 1 9 mg/ml, in contrast to the sober group, which have 31% in this range The fact that urine flow is faster in persons who have taken alcohol means that the time taken to produce a certain volume of urine is shorter, so that more confidence may consequently be placed on the U/B ratios found

The material used was selected according to the criteria given below Out of 1000 consecutive cases, those were chosen in which the blood

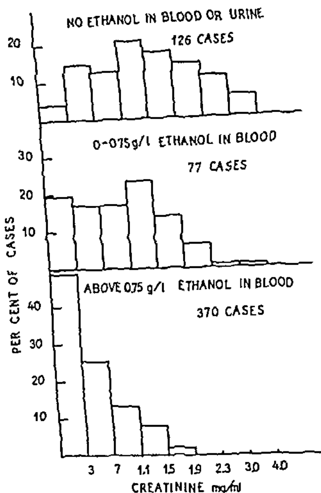


Fig 1 D strib t on of

ethanol conc was above 1 5 g/l, the blood and urine samples were obtained at an interval not exceeding 20 minutes, the volume of urine

reason why a lower limit was chosen for the volume of urine is that experience has shown the loss of ethanol through evaporation frequently to be large when small amounts of urine are voided into a large

container, especially if some time elapses before the urine is transferred to a closed tube

The whole material was divided into three groups and the U/B ratio calculated as shown in table 4

Table 4

The ratio of the concentration of ethanol in urine to that in blood taken at the same time The time between taking blood and urine samples was less than 20 min The volume of urine voided was always between 30 and 300 ml

Number of cases	Range of blood ethanol conc (g/l)	*U/B ratio*	*s*	Range
69	1 50–2 00	1 44	0 1	1 14–1 64
38	2 00–2 50	1 37	0 09	1 16–1 57
46	>2 50	1 33	0 10	1 07–1 55

It is evident that, as the blood ethanol concentration increases, the U/B ratio approaches that obtained in the experimental series

Discussion.

The theory that complete equilibrium is obtained in the kidneys between the water phase of the perfusing blood and the urine produced (WIDMARK 1932) is strongly supported by the investigation described here The ratio of 1 30–1 35, which is in agreement with the figure given by several investigators (SOUTHGATE & CARTER 1926, HAGGARD & GREENBERG 1934, HAGGARD *et al* 1940) corresponds to a concentration of solids in the blood of 23–26% if the urine is taken to be pure water These figures seem to be rather high, on the other hand the U/B ratios found are in agreement with *in vitro* equilibration experiments by HAGGARD, GREENBERG, CARROLL & MILLER (1940), who found values of 1 34, 1 30 and 1 27 for urines of density 1 002, 1 012 and 1 028, respectively The small blank value for urine examined by the Widmark method (generally below 0 05 g/l) will tend to give slightly high values for the U/B ratio

As to the samples from legal cases, it may be of interest to calculate the maximal ratio that could be expected If it is assumed that the rate of urine production is one ml/min and the volume of urine voided 300 ml, then the blood ethanol concentration will have been about 0 75 g/l higher when the urine production began (5 hours before) than at the time the

blood sample was taken If it is also assumed that the rate of urine production has been falling during the period, we may as a reasonable figure assume that the blood ethanol concentration corresponding to the urine sample was 0 5 g/l higher than at the time of withdrawing the blood sample In a subject whose measured blood ethanol was 1 5 g/l we should thus find a U/B ratio of 1 74 If the blood ethanol value was 2 5 g/l we should find the U/B ratio to be about 1 57 In the material shown in table 4, no U/B value above this range was observed When both ethanol and creatinine were determined in the urine, it was noticed that with a high U/B ratio the creatinine concentration was generally also high, indicating a relatively long collecting period

It appears safe to conclude from this work that at some time during the accumulation of a urine sample the blood ethanol concentration has been at least equal to the urine ethanol concentration divided by 1 35

Summary.

1 In 15 experimental subjects the ratio of urine ethanol concentration to blood ethanol concentration was found to 1 35 as an average for all the 55 urine collection periods In two experiments on one subject the urine to serum concentration ratio was found to be 1 07 The sources of error in this kind of experiment are discussed

2 In material from forensic cases submitted for analysis, when blood and urine samples are taken at the same time, the ratio of the ethanol concentration in the urine to that in the blood is shown to approach the theoretical value for diffusion equilibrium when the blood ethanol concentration is so high that the change in blood ethanol during the urine accumulation in the bladder becomes relatively unimportant

3 The results presented strongly support the assumption that the kidneys have no capacity to concentrate ethanol It is concluded that dividing the ethanol concentration in a urine sample by 1 35 gives a figure representing a lower limit for the maximal blood ethanol concentration during the period of urine accumulation

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From the Research Department, AB KABI, Stockholm, Sweden

Amphetamine, Diethylpropion and Tranylcypromin, a Psychopharmacological Study in the Relation between Structure and Activity

By

B Melander and Gisela Glmiecke

(Received May 8 1961)

The chance observation, made during clinical work on the tuberculostatic activity of iproniazid, that the compound had a "mood elevating" effect led to intensive experimental studies, expecially by Zeller and his group, recently reviewed by ZELLER (1960) They found iproniazid to be a potent inhibitor of mono amine oxidase (MAO) The observation of the anti-depressive effect of iproniazid and its possible correlation with MAO-inhibition has resulted in preparation of several so called "psychic energizers" Practically all of them are chemically related to iproniazid they are often referred to as irreversible inhibitors of MAO

While looking for MAO substrate competitors, the Research and Development Division of Smith Kline & French Laboratories[1]) found trans 2 phenylcyclopropylamine to be a potent reversible competitor for MAO The non proprietary generic name adopted in U S A (and the U K) for this compound is tranylcypromin It is also known as SKF 385[2])

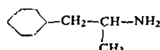
The properties of tranylcypromin have been described by COSTA *et al* (1960) GREEN & SAWYER (1960), HIMWICH *et al* (1960), HORITA & MCGRATH (1960) MAASS & NIMMO (1959), OZAKI *et al* (1960), PLETSCHER (1960) SARKAR *et al* (1960) SPENCER *et al* (1960), TEDESCHI *et al* (1959 a and b 1960) and by ZELLER (1960) There seems to exist a principal difference in mode of action between the hydrazide type compounds and tranylcypromin, in that the hydrazides inhibits the MAO, whereas tranylcypromin competes with the natural substrate for MAO Thus, after

[1]) Philadelphia Pa, U S A.
[2]) Parnate

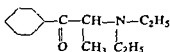
treatment with tranylcypromin the MAO will not have to be re synthesized, but can continue to metabolize its normal substrate as soon as the tranylcypromin has been removed from the organism

Pharmacological studies on man have been reported by HORWITZ *et al* (1960) Clinical studies by AGIN (1960), GOLDMAN (1960), LEMERE (1960) and by LESSE (1960) show tranylcypromin to have anti depressive properties

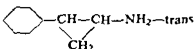
Since tranylcypromin can be considered to be chemically related to two other compounds with activity on the central nervous system recently studied in our laboratories, viz amphetamine and diethylpropion, and discussed by MELANDER (1960), we decided to include tranylcypromin in a comparative study with amphetamine and diethylpropion under the same experimental conditions The structural formulae for amphetamine diethylpropion and tranylcypromin given in figure 1 show that all have a benzene nucleus and a nitrogen atom connected by a carbon carbon chain



Amphetamine



Diethylpropion



Tranylcypromin

Figure 1

Experimental Technique and Results

A *In vitro MAO Competition*

The degree of competition for substrate by the three compounds was studied with tyramine as the substrate in a Warburg apparatus it is compared with that of amphetamine in table 1

B *Motor activity Studies*

Three different techniques have been used to evaluate the increase or decrease in activity due to the test compounds

Table 1.

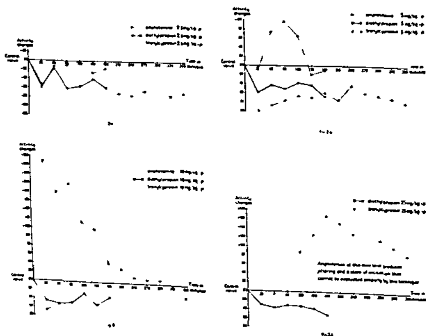
Mono amine oxidase inhibition studied by the Warburg technique
Tyramine substrate Incubation time 30 minutes at pH 7 3 and temperature 37°C.
Effect expressed as the mol/l concentration producing 50% inhibition of MAO = I 50

Compound	I 50 amphetamine/I 50
Amphetamine	1
Diethylpropion	0 08
Tranylcypromin	500

I Photo-cell technique

The design used for this part of the study is the same as described by MELANDER (1960)

In order to ascertain the optimal dose level, dose-response curves were constructed for the three compounds Figure 2 shows the results obtained at dose levels of 2 5 mg/kg i p , 5 mg/kg i p , 10 mg/kg i p and 25 mg/kg i p As demonstrated by MELANDER (1960), the effects produced by amphetamine and diethylpropion do not last longer than 180 minutes These two compounds were therefore not followed for more than 3 hours in



Motor activity by Photo-cell registration

Figure 2

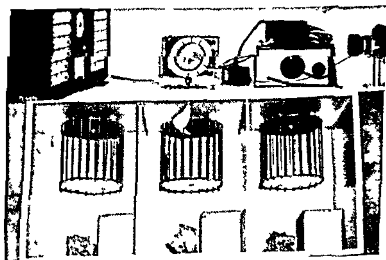
the circumstances Because of a possibly more sustained effect from tranylcypromin, this compound was followed for 6 hours Comparative studies with all three compounds could not be carried out at a higher dose than 10 mg/kg i p , since amphetamine at a dose level of 25 mg/kg i p produced "jittering" in the mice Thus a numerical evaluation increased activity due to the drug was not possible, so that only diethylpropion and tranylcypromin remained for evaluation at the dose level of 25 mg/kg i p

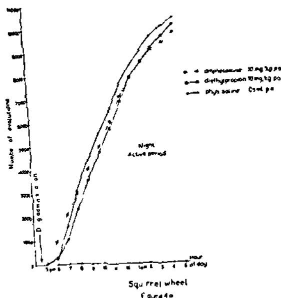
II Squirrel wheel cage

LJUNGBERG (1957) introduced a new variety of the old squirrel wheel technique His apparatus allows the mice to choose between working in a wheel or staying in a little box in another part of the available space STROM (1960), utilizing Ljungberg's set up, found the technique to be of value for evaluating both sedative action and central stimulation

The apparatus built by Mr Calnert State Pharmaceutical Laboratory Stockholm was used in our study to exploit the experience gained by Ljungberg and by Strom The apparatus shown in figure 3 contains 24 individual compartments with housing facilities The number of revolutions for each wheel is recorded separately In order to allow operation of the apparatus independently of normal working routine automatic photographic registration of the accumulated figures every half hour was arranged At the end of an experiment the developed film will supply all necessary data for evaluating the drugs effect

The design of the study was a triple cross over test with physiological saline as the placebo The study was carried out both during the active





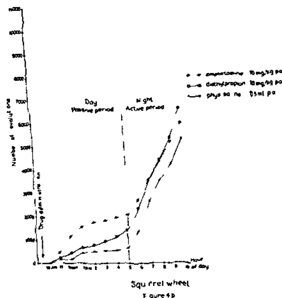
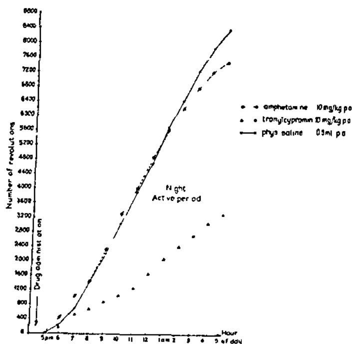


Fig 4a and 4 b Results from triplecross-over test in the squirrel wheel set up Compounds under study amphetamine, diethylpropion and phys saline

period, which is in the dark, and the passive period in daylight Because of the occasional extremely high motor activity observed during the active period, it may be preferable to evaluate the ataractic effect during



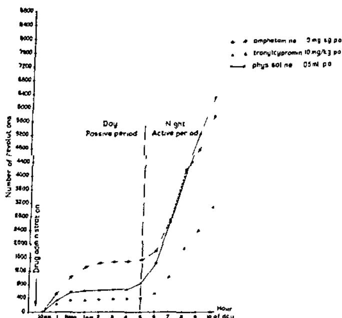


Fig 4c and 4d Results from triple cross-over test in the squirrel-wheel set up Compounds under study amphetamine, tranylcypromin and phys saline

the active period and the presence of activity increasing properties during the passive period By means of the design chosen for this study two series have been run, one with amphetamine, diethylpropion and physiological saline, and the other with amphetamine, tranylcypromin and physiological saline This arrangement offers good possibilities for checking the reproducibility of the test The results obtained are shown in figures 4 a, b, c and d

III Sand sifting

SIEGMUND & WOLF (1952) introduced the sand sifting technique with groups of mice working in the apparatus for the whole test period Adopting this technique, we have chosen to work with another rodent, the Mongolian Gerbil We have also found it useful to run the test as a triple cross over study and to introduce certain modifications of the original technique

An introductory period is used to get the gerbils aquainted with the apparatus Ten animals form a group at each dose level of every compound under test The results are given as the average amounts of sand sifted during the period of active work On the day before the test the animals are trained for 15 minutes every hour for 4 hours The animals are moved from their normal cage into the test cage, into which 150 ml of sand are introduced After the 15 minute work period the animals are moved back to the rest cage

On the day of the test the animals are moved to the test dage for 15 minutes, after which the compound to be tested is administered orally In the control phase physiological saline is administered orally in amounts of 1 ml/100 g of body weight The first working period begins 30 minutes after administration of the compounds The working period is 15 minutes every hour for 6 hours

The degree of activity is given as ml of sand sifted per test period of 15 minutes each hour Figure 5 supplies details of the results

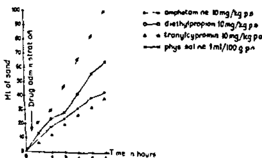
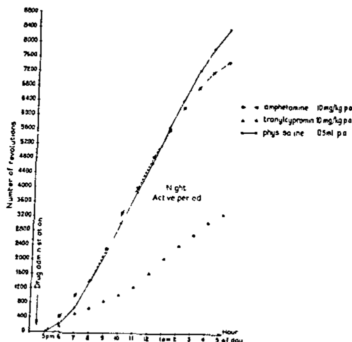


Fig 5 Results obtained by the sand sifting technique in a cross over study of amphetamine diethylpropion tranylcypromin and phys saline



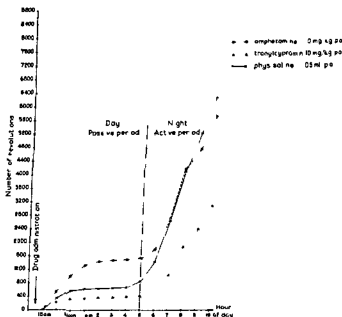


Fig 4c and 4d Results from triple cross-over test in the squirrel wheel set up Compounds under study amphetamine, tranylcypromin and phys saline

To conclude, the three compounds under test have from a psycho-pharmacological point of view to be classified under different headings, with amphetamine showing consistent central stimulation and tranylcypromin a potent MAO-competitor having a predominantly ataractic spectrum Diethylpropion seems to be devoid of both activity stimulating effect and MAO competition This study emphasizes the need for a variety of tests in evaluating psycho pharmacodynamically active drugs

Summary.

The chemically related compounds, amphetamine, diethylpropion and tranylcypromin, have been subjected to a comparative psycho pharmacological study The tests used were

A In vitro mono amino oxidase inhibition
B Motor activity studies,
  I Photocell technique
  II Squirrel wheel cage
  III Sand sifting

In the A situation, only tranylcypromin shows activity of an order placing it among potential "psychic energizers"

In the B I situation, amphetamine consistently increased spontaneous motor activity, whereas diethylpropion consistently reduced activity Tranylcypromin, at the same dose levels as those of amphetamine, decreased motor activity, but at a higher dose level tranylcypromin at first reduced activity but later on quickly increased activity

In the B II situation, amphetamine stimulated the animals whereas diethylpropion and tranylcypromin reduced initiative and spontaneous activity of the animals

In the B III situation, the same pattern as in the B II situation was found

The three compounds under test cannot be classified under one single psycho-pharmacology heading, amphetamine being a stimulant, whereas diethylpropion and tranylcypromin show activity-decreasing properties Only tranylcypromin can be considered a MAO-competitor of potential interest as a ' psychic energizer '

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Discussion.

The results obtained in the experiments clearly show how difficult it is to predict the pharmacodynamic and especially the psycho-pharmacodynamic effects of new chemical compounds related structurally to previously known congeners

An analysis of the results reveals clearly that in comparison with tranylcypromin neither amphetamine nor diethylpropion possesses any merits as MAO competitor, the effect of tranylcypromin being 500 times that of amphetamine and over 6000 that of diethylpropion This in vitro test, however, does not allow of any conclusion as to possible effects on the central nervous system Such an evaluation is preferably made by means of the other tests used in this study

The photo cell technique supplies the information that, when the three tested compounds were run in parallel, only amphetamine increased spontaneous activity whereas both diethylpropion and tranylcypromin decreased spontaneous activity in mice Elevation of the dosage level from 10 mg/kg to 25 mg/kg excluded the possibility of using amphetamine in the comparative study On comparing diethylpropion and tranylcypromin it was found that diethylpropion still depressed activity like an ataractic, whereas tranylcypromin given in the high dose first caused a decrease of activity and then within 1½–2 hours a consistent activity increase that persisted for the whole experimental period

In the squirrel wheel apparatus, both diethylpropion and tranylcypromin showed effects opposite to those of amphetamine Both in the active and passive period – but more pronouncedly in the active period – tranylcypromin decreased activity when compared with amphetamine The activity curves for amphetamine and tranylcypromin lay on either side of the physiological saline placebo values, which shows the absence of any stimulating activity of tranylcypromin at the dose level chosen On comparing amphetamine with diethylpropion the same general pattern for diethylpropion was found as for tranylcypromin, but the differences in the later test were not so pronounced as in the test comparing amphetamine and tranylcypromin

The sand sifting technique once again emphasizes the principal differences between amphetamine on the one hand and tranylcypromin and diethylpropion on the other On studying the results with diethylpropion and tranylcypromin, it is found that the only way of differentiating between the two is by means of the MAO inhibition test Neither of these drugs showed any tendency to the kind of central stimulation that results in an increased motor activity of test animals

Acta pharmacol et toxicol 1961, 18, 249–254

From the Department of Biochemistry and Nutrition Polytechnic Institute Copenhagen

Kidney Damage in Hamsters Receiving an Artifical Diet Containing 5,7-dichloro-8-hydroxychinaldine

By

Frede Christensen and Henrik Dam

(Received May 2 1961)

Hamsters receiving an artificial diet with a high content of sucrose or glucose often develop severe diarrhoea During attempts to prevent this diarrhoea by antibacterial agents, we observed severe kidney damage in all hamsters fed on the basal diet supplemented with 0 1 % of 5,7-dichloro-8 hydroxychinaldine (chlorquinaldol (WHO), chlorchinaldol(NFN), "Sterosan" ®)[1]) for a period of 60 days On repeating the experiment with sterosan, we also examined the effect of a related compound, 5-chloro-7-iodo 8 hydroxychinoline ("Vioform" ®)[2]) under the same conditions Since the observations made in these experiments may be of interest to pharmacologists and may give rise to further studies, they are reported here

Experimental.

The animals were young hamsters, 25–30 days old, of average weight 30–40 g During weaning and until the experiments began, the stock diet (table 1) and water-milk (1 1) were available *ad libitum* Thereafter, the experimental diets and water were given *ad libitum*

The animals were weighed weekly

Three different experiments were conducted

[illegible]

[illegible] was added to diet no 295 as the pure powder, and in group 4 as powdered tablets of Enterovioform ®[3]) The feeding period was maximally 66 days

[1]) Generously furnished by J R Geigy A G Basle Switzerland
[2]) Generously furnished by CIBA Aktiengesellschaft Basle Sweitzerland
[3]) Enterovioform contains sapamine ® as emulsifier

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Table 2

Basal Diet (no 295)

	g
Casein crude	20 0
Sucrose	72 3
Salt mixture U S P No 2 XIII	5 0
Vitamin mixture[1])	0 5
Choline chloride	0 2
Lard[2])	2 0
	100 0

5 mg and sucrose up to 500 mg

[2]) Vitamins A and D_3 were dissolved in the lard Two

A/S Copenhagen) corresponding to 669 i u of vitamin A and 133 i u of vitamin D_3

Influence of sterosan ® and vioform ® on diarrhoea As seen from table 3, 14 of the animals fed on the basal diet in experiment no 1 developed diarrhoea within the experimental feeding period, and one of those fed on the vioform-containing diet had diarrhoea at the end of the experiment In experiment no 3, in which also the vioform-containing diet was used, 5 of the hamsters got diarrhoea after a feeding period of 113–125 days None of the animals fed on the sterosan containing diet developed diarrhoea in experiment no 2, however, in the previous experiment, in which the influence of sterosan on the kidneys was first observed, some of the hamsters receiving this agent also developed diarrhoea



Fig 1 Kidney from a hamster fed on the basal diet with 0 1% of sterosan ® for 60 days (Magnification 4 5)

Experiment no 2 Nine hamsters, 4 males and 5 females, were fed on the basal diet supplemented with 0 1% of sterosan for a period of 81–127 days

Experiment no 3 Seventeen hamsters, 5 males and 12 females, were fed on the basal diet supplemented with 0 1% of vioform for a period of 109–127 days

During the experimental feeding period some of the hamsters developed diarrhoea and died or were killed

Table 1

The stock diet

	g
Alfalfa meal	1
Hog liver powder	3
Salt mixture[1])	3
Ground wheat	8
Ground yellow corn	8
Dried brewers yeast	8
Casein crude	14
Corn starch	27
Sucrose	28
Vitamin E (Ephynal , Roche)	5 mg

1) McCollum Simmonds Salt Mixture no 185 supplemented with 13 mg KI 130 mg $CuSO_4$ 5 H_2O and 394 mg $MnSO_4$ H_2O per 100 g

Results.

The results of the three experiments are shown in Table 3

All the hamsters fed on the sterosan containing diet had severe macroscopic kidney damage, no such lesions were seen in the animals fed on the basal diet or in those fed on the vioform-containing diet However, in experiment no 3, the kidneys in 5 of the 17 hamsters fed on the vioform containing diet for 127 days exhibited a slight to marked pale appearance

Description of the kidney damage The kidneys of the animals from the sterosan-group were pale, with markedly depressed areas on the surface (fig 1) The histological sections, stained with hematoxylin and eosin, showed that the lesions in the kidneys were located in the external part of the cortex The tubular cells were degenerated round the depressed areas of the kidney surface From the depressed surface areas strands of degenerated tubular cells partly penetrated the cortex of the kidneys These strands did not stain with the van Gieson's stain for connective tissue Apparently the glomeruli were not damaged (fig 2) The pale kidneys from the vioform-treated hamsters showed slight tubular changes

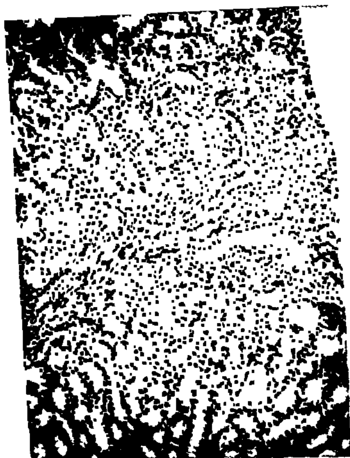


Fig 2 Damaged area in the kidney cortex of a hamster fed on the basal diet with 0 1% of sterosan ® (Haematoxylin eosin, magnification 300)

The question whether microorganisms play a role in the development of the diarrhoea is not settled In previous experiments, diarrhoea occurred in groups of hamsters fed on the high sucrose basal diet supplemented with 0 1% of various antibacterial agents· phthalylsulfathiazole, chlortetracyclin (aureomycin ®), tetracyclin (achromycin ®), erythromycin, or with 0 1% of each of these compounds plus 0 1% phthalylsulfathiazole

The mean gain in weight for the hamsters fed on the sterosan- or the vioform-containing diet for 8 weeks in experiments 1 and 2 was nearly the same, 25–21 g (males–females) and 23–22 g (males–females), respectively The mean weight gain for the males fed on the basal diet in experiment no 1 was 35 g in 6 weeks (group no 1) and 28 g in 8 weeks (group no 2)

Table 3.

Kidney damage and diarrhoea in relation to the experimental diets

Experiment no	Group no	Diet	Kidney damage, diarrhoea and days in experiment	Males					Females					
1	1	Basal (no 295)	Kidney damage		0		0	0	0	0	0			
			Diarrhoea	+	+	+	+	+	+	+	0	+	+	
			Days	50	50	50	50	50	25	32	63	33	36	
	2	Basal	Kidney damage	0	0	0	0	0	0	0			0	
			Diarrhoea	0	0	0	0	0	+	+	+	+	+	
			Days	63	63	63	63	63	33	34	52	33	34	
	3	Basal + 0 1 % of Vioform	Kidney damage	0	0	0	0	0	0		0	0	0	
			Diarrhoea	0	0	0	0	0	0	+	0	0	0	
			Days	62	62	62	62	62	62	62	62	62	62	
	4	Basal + 0 1 % of Enterovioform[2]	Kidney damage	0	0	0	0	0	0	0	0	0	0	
			Diarrhoea	0	0	0	0	0	0	0	0	0	0	
			Days	66	66	66	66	66	66	66	66	66	66	
2		Basal + 0 1 % of Sterosan	Kidney damage	+	+	+	+		+	+	+	+	+	
			Diarrhoea	0	0	0	0		0	0	0	0	0	
			Days	81	81	81	81		81	81	127	127	127	
3		Basal + 0 1 % of Vioform	Kidney damage	0	0	0	0	0[1]	0	0[1]	0[1]	0[1]	0[1]	0
			Diarrhoea	0	0	0	0	0	0	0	0	0	0	+
			Days	127	127	127	127	127	109	127	127	127	127	115
		Basal + 0 1 % of Vioform	Kidney damage						0	0	0	0	0	0
			Diarrhoea						+	+	+	+	0	0
			Days						120	125	113	120	127	109

No macroscopic structural lesions were seen, but the kidneys had a more or less pale appearance
As powdered tablets

Acta pharmacol et toxicol 1961, 18, 255–262

From the Department of Pharmacology, University of Turku

Inhibition by Methysergid of 5-Hydroxytryptophan Toxicity to Mice

By

J Kärjä, N T. Kärki and E. Tala

(Received July 18 1961)

5 Hydroxytryptophan (5HTP) is the precursor of 5 hydroxytryptamine (enteramine - serotonin 5, HT) in the organism The administration of 5HTP increases the amounts of 5HT in most tissues (UDENFRIEND *et al* 1957, DAVIDSON *et al* 1957, PAASONEN & GIARMAN 1958) Monoamine oxidase (MAO) plays an important part in the physiological inactivation of 5HT (BLASCHKO 1952, FREYBURGER *et al* 1952, SJOERDSMA *et al* 1955), and MAO-inhibition increases the level of 5HT, particularly in brain (UDENFRIEND *et al* 1957, PAASONEN & GIARMAN 1958, PLETSCHER *et al* 1959) If 5HTP is given to animals previously treated with MAO-inhibitor, there is an increased accumulation of 5HT in tissues and in the reactions induced by 5HTP (BOGDANSKI *et al* 1958, HORITA & GOGERTY 1958, PAASONEN *et al* 1961)

During recent years several drugs have been introduced that antagonise 5HT in vitro and in vivo In order to study the effectiveness of one of the most recent, methysergid (WHO, D C I) N (1 (hydroxymethyl)-propyl)-4-methyl (+)-lysergamid, deseril ®, we have investigated its action on the toxicity of 5HTP to MAO inhibited mice In addition, the influence of methysergid on the pyrogenic and motor activity depressing actions of 5HTP administration has been studied

Material and Methods

Female albino mice weighing 18 to 21 g were used

They were pretreated with 5 mg/kg of pheniprazine (PIH, β-phenylisopropylhydrazinehydrochloride) subcutaneously during the morning After 6 hrs they were given intraperitoneally from 50 to 1000 mg/kg D L-5 hydroxytryptophan in a 1–5% (w/v)

Discussion.

It has previously been found that hamsters of the same age as those used in the present experiment eat 4–6 g of the basal diet per animal per day If the addition of vioform ® or sterosan ® does not influence the eating habits of the animals, the daily ingestion of each of the two antibacterial agents was about 5 mg per animal, corresponding to about 10 mg per 100 g of body weight. The doses used in human therapy are considerably lower, e. g , 600 mg of sterosan or 1,500 mg of vioform per day for adults with diarrhoea. These amounts correspond approximately to 1 mg of sterosan or 2.5 mg of vioform per 100 g of body weight, and the treatment is usually not continued for a period of time comparable to that used in our experiments. Therefore, the results of the experiments reported here do not necessarily imply a risk of kidney damage from ordinary thera peutic application of the drugs studied

Further, in other experiments with hamsters fed on the artificial basal diet (no. 295), pale kidneys were occasionally observed These kidney changes may have been caused by particular properties of the diet, such as the low content of essential fatty acids or the high content of sucrose or both

Kidney changes, somewhat similar to those observed in the present experiments, have been noticed in rats fed for 26 weeks on fat-free diets or on diets containing hydrogenated arachis oil as the sole source of fat (FUNCH, AAES-JORGENSEN & DAM 1957) It is possible, therefore, that in the experiments here recorded a latent abnormality of the tissue has rendered the kidney tubuli particularly sensitive to a noxious effect of certain drugs. These problems should be studied further in hamsters receiving other, and especially more normal diets as well as in other species

Summary.

Severe kidney lesions were observed in hamsters fed on an artificial diet supplemented with 0 1% of 5,7-dichloro-8-hydroxychinaldine (sterosan ®) for 81 days or more No such lesions were seen in hamsters fed on the same basal diet supplemented with 0 1% 5-chloro-7-iodo-8-hydroxychinoline (vioform ®) for as long as 127 days or in those fed on the unsupplemented basal diet

The kidney lesions were found in the tubuli cells of the cortex. Apparently the glomeruli were not affected

It is possible that the artificial basal diet used in these experiments has increased the sensitivity of the tubuli cells to the damaging effect of certain drugs.

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Table 2

The effect of methysergid on the temperature of 5HTP and phenіprazine treated mice Each group consisted of *10 animals The mean change (± s e m)* in rectal temperature from the initial level

Dose of 5HTP	M = Methysergid C = Control	Temperature at different times (hrs) after 5HTP injection and the values of P between M and C groups and in some instances between two M or two C groups									
		½	P	1	P	1½	P	2	P	4	P
50	M	−0 9 ±0 51	<0 001	+0 6 ±0 32	<0 001	−0 5 ±0 24	<0 001	−0 9 ±0 37	<0 001	+0 9 ±0 17	
	C	+1 9 ±0 22		+2 2 ±0 14		+1 7 ±0 35		+1 7 ±0 22		+0 6 ±0 28	
100	M	−0 3 ±0 56	<0 001	+2 9 ±0 56		+0 9 ±0 77	<0 01	+0 3 ±0 72		+0 1 ±0 83	
	C	+3 7 ±0 33		+3 0 ±0 46	<0 001	+3 3 ±0 42		+1 4 ±0 81	<0 001	+0 2 ±0 84	=<0 001
300	M	+1 9 ±0 51	<0 001	+1 3 ±0 28		+0 1 ±0 49		+0 7 ±0 80		−0 4 ±0 49	
	C	+2 1 ±0 31		+1 3 ±0 49		−0 5 ±0 79		−1 1 ±0 92		−2 2 ±0 56	
500	M	+3 0 ±0 34	<0 001	+2 5 ±0 22		+0 9 ±0 38		+1 1 ±0 68	<0 01	−2 2 ±0 64	
	C	+1 1 ±0 72		+1 4 ±0 36		−0 4 ±0 87		−2 6 ±1 02		−5 1 ±1 40	

solution in 0 01 N-HCl, made immediately before use One hour before and one hour after 5HTP administration the test animals were given a subcutaneous dose of the possible antagonising substance The control animals were injected with pheniprazine and 5HTP at the same time, but saline was injected instead of the antagonising substance The results of the toxicity tests are based on mortality at 12 hrs and the LD50's are calculated

The rectal temperature was measured with an electric thermocouple (Thermorapid) at intervals of 30 min for 4 hrs after 5HTP injection The tests were conducted at room temperature, 21 to 23°C As a rough measurement of motor activation by methysergid, tracings were obtained by placing treated and control animals for one minute on the smoked paper under a glass cylinder

Table 1.

The protective effect of methysergid on 12 hr lethality of 5HTP in pheniprazine (5 mg/kg) pretreated mice Each group consisted of 10 animals

5HTP mg/kg	Lethality percent	
	Methysergid 5 mg/kg	Control
50	0	0
100	0	20
200	10	20
300	20	50
500	30	70
1000	80	–

Results.

The effect of methysergid on mortality The results of the toxicity study are summarised in table 1 The 12 hrs LD50 of i p 5HTP in pheniprazine-treated mice was 305 mg/kg When 5 mg/kg of methysergid were given one hour before and one hour after 5HTP, there was a reduction in death rate and the LD50 rose to 625 mg/kg Most deaths occurred within 6 hrs of 5HTP administration, animals that lived for 12 hrs survived

A lower dose (0 5 mg/kg) of methysergid also proved to have a protective action, but a dose of 0 1 mg/kg was ineffective in our experiments

The effect on pyrogenic action When temperatures of test animals were followed, it was soon noted that the effect of 5HTP on rectal temperature was dependent on the dosage used In control animals receiving smaller doses, 50 to 100 mg/kg of 5HTP, there was a temperature increase lasting several hours with a maximum at 1½ hrs after administering the drug (table 2) Larger doses (300–500 mg/kg) also induced an initial elevation of temperature, which subsided after 1½ hrs, the animals becoming hypothermic

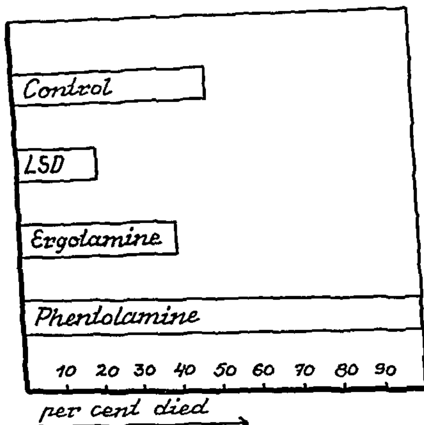


Fig 2 Comparison of effects of phentolamine, lysergid (LSD) and ergotamine on toxicity of 5HTP Each group consisted of 20 mice, all given pheniprazine 5 mg/kg s c and 300 mg/kg of 5HTP i p

Control group no other treatment
Lysergid group 1 mg/kg LSD25 s c
Ergotamine group 1 mg/kg ergotamine tartrate s c
Phentolamine group 5 mg/kg phentolamine s c

Pheniprazine alone at the dosage level used had little or no effect on temperature Pheniprazine and methysergid given together, however, slightly reduced the temperature of test animals

Behaviour Administration of 5HTP produced within 4 to 5 min a pronounced tremor and stiffness of the limbs and later on convulsions Methysergid reduced these signs, as shown in fig 1

Effect of other antagonists Lysergic acid diethylamide = lysergid (LSD) and ergotamine in doses of 1 mg/kg each also had a protecting effect on 5HTP-induced mortality, as would be expected (fig 2) On the other hand, administration of phentolamine, known to be an effective

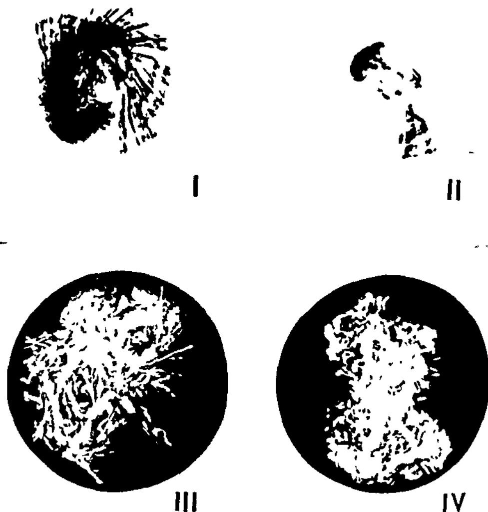


Fig 1 The influence of methysergid on motor depression The animals had received phenіprazine 5 mg/kg and 5HTP 300 mg/kg
Control animal (I and II) No other treatment ½ hr (I) and 1 hr (II) after 5HTP injection (1 min)
Methysergid animal (III and IV) Methysergid 5 mg/kg twice ½ hr (III) and 1 hr (IV) after 5HTP injection (1 min)

Methysergid effectively inhibited the pyrogenic reaction of small doses of 5HTP. When large doses of 5HTP were used, methysergid treatment prevented the fall in temperature. This effect was clear with 500 mg/kg of 5HTP; the mean temperature of methysergid-treated mice remained higher than that of controls; after 2 and 4 hrs. the difference was about 3°.

blocking of noradrenaline effects may lead to more disturbed balance, which thus could be the reason for the higher mortality

The finding that after large doses of 5HTP methysergid had a temperature maintaining effect was unexpected However, it is known that large doses of 5HTP, especially when given with a MAO inhibitor, produce peripheral vasodilatation (BOGDANSKI *et al* 1958), which evokes increased loss of heat Possibly methysergid, by preventing this vasodilatation, enabled the animal to maintain higher temperature

Although behaviour was only observed visually and by rough measurement, there was no doubt about the relieving action of methysergid on 5HTP induced tremor and stiffness in the test animals

Summary.

The lysergic acid derivate, methysergid, was able to decrease the toxicity of 5 hydroxytryptophan in mice pretreated with the monoamine oxidase inhibitor, phenipraziné Lysergic acid diethylamide (lysergid) and ergotamine also decreased the toxicity of 5 hydroxytryptophan, but phentolamine had an opposite effect

The pyrogenic action of small doses of 5 hydroxytryptophan was reduced and the temperature depressing effect of high doses was counteracted, by methysergid

There was an indication that methysergid reduced the depression of motor activity produced by large doses of 5 hydroxytryptophan

Acknowledgement

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sympatholytic agent at a dose of 5 mg/kg increased the toxicity of 5HTP by 100% Lysergid and ergotamine had a methysergid-like action on the pyrogenic effect of 5HTP.

Discussion.

FANCHAMPS *et al* (1960) have shown *methysergid an effective 5HT* antagonist in several test preparations They found that methysergid treated mice survived a dose of 210 mg/kg 5HT, an amount producing 50% mortality in untreated animals On the other hand, it has been postulated that peripherally administered 5HT passes with difficulty across the bloodbrain barrier (UDENFRIEND *et al* 1957, SHORE *et al* 1957, KARKI & PAASONEN 1959), and one could therefore assume that effects of 5HT administered by this means are easy to antagonise However, 5HTP has been found to penetrate rapidly into tissues, where it is decarboxylated to 5HT If a MAO-inhibitor is given before 5HTP, a rapid and marked accumulation is found in many tissues, we are thus in a position to study the effects of endogenously formed 5HT (UDENFRIEND *et al* 1957, BOGDANSKI *et al* 1958, HORITA & GOGERTY 1958, PAASONEN *et al* 1961)

In our experiments on mice it was shown that methysergid could reduce pheniprazine-5HTP mortality which apparently was due to a high 5HT level

The dosage of methysergid (5 mg/kg twice) used by us was rather high compared with that recorded by FANCHAMPS *et al* (1960) to be effective in preventing the effects of 5HT in the rat Since the toxicity of methysergid is low (FANCHAMPS *et al* 1960) *and its duration of action* rather short (BERDE *et al* 1960), we chose this dose to make sure of the methysergid action A lower dose (0 5 mg/kg) was also effective

Several other lysergic acid derivates have anti-5HT abilities (ROTHLIN 1957) Lysergid (LSD) and ergotamine, as would be expected, reduced the toxicity *Some of the pharmacological effects of lysergid and* 5HTP have been shown to be additive (HORITA & GOGERTY 1958, BOGDANSKI *et al* 1958) this was not true of our toxicity experiments

We consider as evidence that the protective effect of lysergic acid derivates against 5HTP toxicity is not connected with their sympatholytic properties the fact that phentolamine, which is known to be a strong adrenergic blocking drug, did not reduce mortality from 5HTP, on the contrary, an enhancement was noted

In this connection it is interesting to keep in mind BRODIE's (1958) hypothesis about the role of 5HT and noradrenaline in brain function According to him, 5HT and noradrenaline are mediators of opposing parts of the central nervous system With phentolamine administration,

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α-Keto Acids in β-Aminopropionitrile Intoxication (Lathyrism)

By

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(Received July 18 1961)

This work was undertaken because of some findings on experimental lathyrism, namely, that the "carbonyl fixatives", iproniazid and semicarbazide either aggravate the symptoms of experimental lathyrism (JUVA, MIKKONEN, TUOMINEN & KULONEN 1959, ROY, LIPTON, STRONG & BIRD 1959) or cause similar effects when given alone (NEUMANN, MAXWELL & McCOY 1956) Experimental lathyrism is clearly influenced by the amount of amino acids in the diet, without regard to the composition of the amino acid mixture (JUVA *et al* 1961) Thus the general intermediary metabolism of amino acids, and consequently of keto acids, is involved in β aminopropionitrile poisoning We were also interested to find out whether any unusual carbonyl derivates were present in the tissues of lathyritic animals

Experimental.

[illegible]

amount of sodium sulphate In some experiments both groups of eggs received additionally 10 mg of semicarbazide hydrochloride (Merck pro analysi) to trap carbonyl compounds After 4 hours of continued hatching, the embryos were harvested weighed and homogenized in 10 ml of water The homogenate was treated as described by RICHTER (1937), and the resulting 2 4-dinitrophenyl hydrazone mixture chromatographed by KULONEN, CARPÉN & RUOKOLAINEN's procedure (1952)

Rats Wistar rats received the lathyritic (sweet pea) or control diets described earlier (JUVA *et al* 1961) To decrease variation four rats were treated as a group, and three groups were on each diet In the first experiment (average weight of the rats at the beginning 62 g, after 29 days experiment 87 g in the lathyrus group and 112 g in

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The variations are large but the results for urine are in satisfactory agreement with the findings of KRUSIUS (1940) From adult rats he obtained an excretion of α ketoglutaric acid of 0 07 mg/12 hr on a high fat diet and 0 47 mg/12 hr on a high carbohydrate diet For the excretion of pyruvic acid the range on different diets was still larger 0 03 1 77 mg/12 hr The blood values are in a reasonable agreement with those for man (KULONEN *et al* 1952) We are unable to explain the large difference in α ketoglutaric excretion between the first and second experiments In the first the animals were larger but this does not seem a sufficient explanation We are rather inclined to believe that the values for the first experiment are unreliable because of contamination of the samples with faeces

Since the different experiments point in the same direction we suggest that the concentrations of pyruvic acid and to a less extent of α keto glutaric acid are increased in lathyrism both in embryonic tissue and in blood but that their urinary excretion is decreased Considering the smaller bodyweight of the intoxicated animals the difference in the excretion is scarcely of metabolic consequence However this is not certain since excretion seems to decrease immediately after the first consumption of the lathyrus diet

There was no obvious qualitative difference in the keto acids between normal and lathyritic tissues Some possible intermediates for example the 2 4 dinitrophenylhydrazone of the glutamic semialdehyde may have migrated in chromatograms so near to the front that they escaped atten tion

Discussion

The best known cause for the increase in keto acids of the tissues is an inhibition of the oxidative decarboxylation because of a shortage of th amine Nothing is known about oxidative decarboxylation in lathyritic tissue or in the presence of β aminopropionitrile We have conducted some *in vitro* experiments on the consumption of oxygen in liver slices with pyruvic acid as substrate A slight decrease was noted in the presence of β aminoprop onitrile but first only at a concentration of 10^{-2} M The neutral fat of rat skin is markedly decreased in lathyrism but it is not known whether the conversion of the pyruvic acid to fats is impaired

The other possible reason for the increase in keto acids may be disturbed transamination The amination of pyruvate is depressed by all enzyme inhibitors blocking intermediate steps from pyruvate to ketoglutarate via the citric acid cycle (BRAUNSTEIN & AZARKH 1957 a) In isoniazid drugged animals the transamination of the α keto acids is also depressed (BRAUN STEIN & AZARKH 1957 b) *In vitro*-experiments (with T NIKKARI) to

the control group) the cages were placed above large funnels containing a wire net to retain faeces Sulphuric acid was put beforehand in the receiving beakers to inhibit microbial growth in the urine In the second experiment the animals were a little smaller (at the beginning 46 g, after 23 days on the lathyrus diet 63 g and on the control diet 80 g) To collect the urine, the falling faeces were diverted outside the recipient beaker by a glass spindle placed below the funnel, and contamination with faeces was thus avoided Toluene was added to inhibit infection For analysis, the urines of 3–4 consecutive days were pooled The animals were then killed by decapitation, and the blood was collected into weighed centrifuge tubes The blood and urine samples were deproteinised with tungstic acid, and the supernatants were treated with 2,4-dinitrophenylhydrazine solution (KRUSIUS 1940) The hydrazones of the acid oxo compounds of blood and urine were then prepared and analyzed as indicated above (KULONEN *et al* 1952)

Results.

The final results are collected in table 1 The statistical significance of differences was calculated by the *t*-test The urine concentrations were calculated as daily averages, and simultaneous lathyritic and control samples were compared

Table 1

[illegible] ketoglutaric [illegible] samples also [illegible]

Sample		α Ketoglutaric acid		
		Control	Treated	P
Chick embryos, µg/g				
without SC		11 9 ± 4 8 (6)	14 5 ± 4 6 (6)	NS
with SC		16 5 ± 7 0 (4)	15 2 ± 3 5 (5)	NS
Rat blood, µg/g	exp 1	1 6 ± 1 0 (13)	2 0 ± 0 9 (12)	NS
	exp 2	–		–
Rat urine[1]) µg/24 hr				
	exp 1	1490 ± 450	460 ± 340	<0 001
	exp 2	200 ± 150	100 ± 56	<0 05
		Pyruvic acid		
		Control	Treated	P
Chick embryos, µg/g				
without SC		5 7 ± 1 9 (6)	9 1 ± 2 8 (6)	<0 05
with SC		17 4 ± 9 7 (4)	25 0 ± 5 5 (5)	NS
Rat blood, µg/g	exp 1	4 2 ± 2 0 (9)	5 8 ± 2 1 (5)	NS
	exp 2	5 0 ± 2 0(11)	5 6 ± 2 3(12)	NS
Rat urine[1]) µg/24 hr				
	exp 1	67 ± 10	48 ± 15	NS
	exp 2	86 ± 39	58 ± 17	NS

1) for experimental details see the text

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Effects and Fate of Lipoperoxides in Chicks and Rats

By

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(Received June 22, 1961)

A previous paper from this laboratory described the fate of orally ingested lipoperoxides (GLAVIND & TRYDING 1960) Peroxide-containing fatty acid esters were found to be partly transformed by the action of pancreatic juice Peroxide-containing free fatty acids were formed, and the total quantity of peroxides decreased, although they were not completely eliminated When lipoperoxides were given to rats with cannulated thoracic ducts, no lipoperoxide could be recovered in the lymph It was concluded that the essential site of destruction of orally injected lipoperoxides is probably the intestinal mucosa

The purpose of the work described in this paper was a study of the fate of lipoperoxides given intravenously or intraperitoneally

Materials and Methods.

[illegible] or tween 80 ®, were dissolved together in ether and the ether was evaposated and the residue finely dispersed in 0 9% saline by means of a tissue disintegrator

Experimental animals Day-old chicks (New Hampshire × White Leghorn) were fed until the age of at least 1 month, on one of three *diets*

reproduce with β-aminopropionitrile the effect of *isoniazid* on the liver amino-transferase were not successful

The addition of semicarbazide, which itself has an effect similar to that of lathyrism, increased the concentration of pyruvic acid three fold but did not affect the α-ketoglutaric acid The difference between the control and lathyritic sample also persisted The results from the experiment with semicarbazide were not accurate enough to permit conclusions about the mechanism of the increase in pyruvic acid, *i e* to decide between increased formation or decreased breakdown

Summary.

The pyruvic and α-ketoglutaric acid concentrations were moderately increased in β-aminopropionitrile treated chick embryos and in the blood of lathyritic rats

Acknowledgements

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or hours Sometimes a dark coloration of the lungs was found at autopsy, suggesting the formation of methaemoglobin

Injection of about 1 μeq of peroxidized ethyl linoleate per g body weight was sometimes also fatal, and caured a dark coloration of the lungs

The other peroxide containing products, cholesterol free fatty acids and phospholipids, were given in relatively small amounts, and no evidence of toxicity was observed

Among the animals injected were a few chicks reared on a vitamin E deficient diet Peroxide containing triglycerides (about 1 μeq /g body weight) were given to three such chicks and ethyl linoleate peroxide (0 6 μeq /g) to two others The injections were well tolerated, the vitamin E deficient animals did not seem to be more sensitive than the normal animals to injections of peroxides It should be mentioned that none of the symptoms seen in chicks kept on a vitamin E free diet (e g , encephalomalacia) were ever observed as a consequence of the injections, whether in vitamin E deficient or in normal animals

Lipoperoxides also were given to a number of rats intraperitoneally The same picture appeared as was obtained with i v injections 5 μeq of triglyceride peroxide per g body weight were well tolerated, whereas ethyl linoleate and butyl peroxides were toxic, although less so by i p than by i v injection Further the death occurred of one rat that had been given per g body weight 2 μeq of peroxide containing cholesterol emulsified with lecithin

Ascites and inflammation of the abdominal wall and the surface of the liver were a regular finding on autopsy of the animals that had died after p i administration of peroxide-containing lipids The same picture was observed when the animals that had received i p injections of peroxide containing triglycerides were killed next day and autopsied

Several rats were given large doses of peroxide-containing triglycerides, either i v or i p , and killed 7 weeks later On autopsy, no pathological findings were recorded

Results of the peroxide determinations on blood, heart, kidneys spleen, lungs and liver of chicks and rats are given in tables 1–4 Some other organs were occasionally examined

Since some of the autopsies were suggestive of methaemoglobin formation methaemoglobin was determined in blood samples taken from several rats that had received various peroxides by i v or i p injection The results are given in table 5

Discussion

The results given in table 1 show that, although the greater part of intravenously injected lipoperoxides generally disappears rapidly from

Normal A commercial starter ration

E free A vitamin E-free but not encephalomalacia producing diet of the composition 30 g crude casein, 3 g gelatine, 10 g hydrogenated palm kernel oil 5 17 g salt mixture (DAM & SONDERGAARD 1953) 0 1 g vitamin mixture (DAM & SONDERGAARD 1953), 0 2 g choline chloride, 51 53 g corn starch, 1 mg vitamin K substitute Vitamins A and D_3 were given in the form of 0 1 ml of an aqueous solution twice a week (DAM HARTMANN, JACOBSEN & SONDERGAARD 1957)

Control 100 g E free diet supplemented with 10 mg d,l-α tocopherol acetate

Adult female rats were obtained from a local dealer and fed on a stock diet (CHRISTENSEN DAM & PRANGE 1952), supplemented with 5 mg d l-α tocopherol acetate per 100 g

Injections and blood sampling The emulsions in amounts of 2–10 ml were injected slowly over 1–4 minutes into the right jugular vein of chicks or anaesthetized rats. From the left jugular vein 1–2 ml blood samples were taken with a syringe in the presence of a little heparin Several rats received the emulsion by intraperitoneal injection To prevent infection from the fur, the skin was opened before the injections were carried out

Determination of lipoperoxides The blood samples and the organs taken at autopsy were extracted in a mechanical disintegrator with 50–100 ml chloroform in the presence of anhydrous sodium sulphate The mixture was filtered and the volume read The whole filtrate, or measured part of it was evaporated and taken into 5 ml chloroform An equal volume of 6% ammonium thiocyanate in ethanol was added and the determination of peroxide was carried out as described previously (GLAVIND & HARTMANN 1955) with exclusion of oxygen The photometric reading was corrected for a reagent blank and for the color of the extract The latter was determined after decolorizing the ferric thiocyanate by adding a drop of a concentrated solution of ascorbic acid in dilute ethanol

Determination of methaemoglobin and total haemoglobin A method essentially identical with that of EVELYN & MALLOY (1938) was used

Results.

Intravenous injections were given to about 35 chicks and 30 rats The phospholipid-emulsions were generally well tolerated A few animals died from thrombosis in or close to the heart, as revealed by autopsy, probably caused by an unsatisfactory state of the emulsion or by the thromboplastic action of the phospholipids Tween 80 ® produced more perfect emulsions than the phospholipids, but had to be used in larger amounts for a given amount of lipid Amounts of Tween 80 exceeding 200 mg proved to be fatal to rats weighing 200–250 g Among the peroxide-containing products, cholesterol gave the least stable emulsions

Peroxide-containing triglycerides, even in amounts as great as 3 μeq per g of animal weight, were well tolerated, and no evidence of toxicity was observed

When, however, *t*-butyl hydroperoxide was injected, amounts of about 1 μeq per g body weight killed the animals in the course of a few minutes

Table 3

Recovery of various intravenously injected peroxides from organs of the chick
(Animals killed one hour after the injection)

Peroxide containing product	Dose μeq/g body weight	Emulsifier	Diet	Peroxide recovery from					
				Blood	Heart	Kidneys	Spleen	Lungs	Liver
Ethyl lineoleate	0 5	Lecithin	Control	2 0	0 04	0 04	0 04	0 9	2 7
–	0 6	–	E free	1 0	0 02	0 07	0 04	1 2	4 5
	0 6	Tween	Control	1 4	0 04	0 06	0 06	2 1	1 1
–	0 6		E free		0 04	0 06	0 11	0 4	1 0
Phospholipid	0 23	None	Normal	6	0 01	0 08	0 52	0 09	1 6
–	0 19		Normal	4 4	0 02	0 05	0 7	0 08	1 3
	0 5		Control	5 5	0 04	0 06	0 45	0 16	2.5
Free fatty acids	0 14	Tween	Normal	1 6	0 03	0 02	0 01	0 08	0 1
–	0 14		Normal	2 5	0 01	0 02	0 02	0 03	0 05
Cholesterol	0 09		Normal	4 1	0 14	0 23	0 7	1 1	0 9
	0 15	–	Normal	3 0	0 05	0 08	0 06	0 2	0 2
Butyl hydroperoxide	1		Normal	0	0	0	0	0	0

Table 4

Recovery of injected peroxides from organs of the rat
(Lecithin as emulsifier)

Peroxide-containing product	Dose μeq/g body weight	Mode of injection	Hours from injection to autopsy	Peroxide recovery from					
				Blood	Heart	Kidneys	Spleen	Lungs	Liver
Triglyceride	1	i v	1	1 25	0 2	0 2	1 2	19	17
	1		1	2 5	0 2	0 4	0 8	24	18
	4 5	i p	24	0 03	0 00	0 01	0 04	0 03	0 8
	3 5		24	0 03	0 01	0 03	0 05	0 06	0 6
Ethyl linoleate	5		3	0 02	0 00	0 01	0 01	0 01	0 07
	7		3	0 01	0 00	0 01	0 05	0 00	0 13

the blood, considerable quantities may still circulate after 1 hour The rate of disappearance of lipoperoxides from the blood stream depends on the chemical nature of the peroxide-containing product and the character of the emulsion The more perfect emulsions of peroxide-containing triglycerides prepared with tween 80 ® remained in the blood much longer than those prepared with phospholipids as emulsifier, and it appears ... which occur as norm... time than peroxide... only minor

Table 1.

Recovery of intravenously injected peroxides from the blood of chicks and rats

Peroxide containing product	Dose µeq /g body weight	Emulsifier	Diet	Percentage recovery from blood after		
				about 2 min	15 min	60 min
			Chicks			
Triglyceride	3	Lecithin	Control	18	8	2.6
–	1	–	–	6 5	3	1 0
–	1	–	E free	8	5	1 3
–	0 2	Tween	Normal	40	37	18
–	0 2	–	–	68	66	44
Ethyl linoleate	0 6	–	Control	14	3	1 4
–	0 6	–	E free	7	5	
Phospholipid	0 23	None	Normal	22	16	6
–	0 19	–	Control	13	10	5
Free fatty acid	0 14	Tween	Normal	4 3	3 7	1 6
–	0 14	–	–	2 7	3 2	2 5
Cholesterol	0 09	–	–	12	8	4
–	0 15	–	–	6	3	3
Butyl hydroperoxide	1	–	–	1 0	0	0
			Rats			
Triglyceride	1	Lecithin	Stock	11	5 5	1 2
–	1	–	–	26	11	2 5

The percentages were calculated from the amounts found in samples of 1–2 ml blood, assuming a blood volume of 8 3 % of the body weight in chicks, 6 7 % in rats

Table 2

Recovery of intravenously injected triglyceride peroxides from organs of the chick

Dose µeq /g body weight	Emulsifier	Diet	Hours from injection to autopsy	Peroxide recovery from					
				Blood	Heart	Kidneys	Spleen	Lungs	Liver
3	Lecithin	Normal	24	1 6	0 17	0 31	3 2	9	7
3	–	–	24	4	0 13	0 4	2 7	10	6
3			48	0 2	0 08	0 2	1 2	6	2 5
3	–	–	48	0 2	0 07	0 2	1 7	9	3
3	–	–	500	0	0	0	0	0	0
3	–	Control	1	3	0 04	0 2	0 3	35	10
3	–	E free	0[1])	17	0 06	0 3	0 3	38	13
1 8	–	Control	1	1 3	0 2	0 14	0 14	25	6
1 7	–	E free	1	1 0	0 3	0 15	0 17	25	8
0 9	–	Control	0[1])	12	0 1	0 13	0 11	11	4
0 9	–	E free	0[1])		0 1		0 10	21	3
0 25	–	Normal	1	0 3	0 15	0 3		24	2 5
0 2	–	–	1[2])	0 6	0 07	0 06	0 10	25	1 1
0 25	Tween	–	1	44	0 35	0 6	2 0	2 7	1 6
0 18	–	–	1	18	0 45	0 44	0 24	1 4	0 6
0 18	–	–	24	8	0 08		0 4	0 5	1 7

[illegible] lar vein, the animal [illegible] is were carried out

When peroxide-containing triglycerides were injected intravenously into the rat, the same picture was obtained as with the chick (table 4) After intraperitoneal injections into rats, much smaller amounts could be recovered from most organs and from blood

The overall picture of *the fate* of parenterally injected lipoperoxides seems to be that intraperitoneally injected triglyceride peroxides are transported by the blood, especially to the liver, to a lesser extent to lungs and spleen and probably to the reticulo-endothelial system elsewhere in the body, where they are metabolized Intravenously injected triglyceride peroxides are also in all probability deposited in the reticulo-endothelial system Imperfect emulsions probably are to a great extent deposited extracellularly, preferentially in the lung capillaries Later, the extracellular deposits are cleared by the blood stream The few observations with other lipoperoxides show that relatively smaller amounts were recovered, especially from the lungs The mechanisms by which these peroxides are eliminated may be different from that for triglyceride peroxides

The elimination of peroxide groups bound to lipids is not a very fast process, since after intraperitoneal application of lipoperoxides, slight amounts can be found in the liver, it appears that a transitory deposition of such peroxides can take place, even intracellularly These results may well seem surprising in view of the great reactivity of the peroxide group

It is known that *peroxide determinations* carried out on animal tissues result in only partial recovery The problem has been previously studied in this laboratory (GLAVIND & HARTMANN 1961) Fresh animal organs were extracted with chloroform containing known amounts of peroxide, peroxide determinations were carried out as described in this paper and the recoveries calculated When liver was extracted, quantities up to 0 1 µeq /g tissue could not be recovered Other tissues destroyed similar quantities but some tissues such as blood, showed a fairly good recovery It is not known whether the peroxide destroying principles interfering with the determination are also active *in vivo*, but evidently they preclude an exact quantitative estimation of the total amount of peroxides present in the tissues This source of error must be even more important when small amounts of peroxides are injected In the experiments reported here doses of 0 1–3 µeq per g body weight were given Since the peroxides were deposited preferentially in lungs, liver and spleen, it is, however, likely that the disappearance of peroxides during the determination is only of minor importance for these organs

Further difficulties in the determination of the total amount of peroxide present in the animal were that not all organs were examined and that the blood volume was not exactly known The total quantity of peroxide in the blood was estimated on the basis of the approximate values for

Table 5.

Methaemoglobin content of rat blood after injection of peroxides.
(Lecithin used as emulsifier)

Peroxide containing product	Dose μeq /g body weight	Mode of injection	Time after injection	Methaemoglob n per cent of total haemoglobin
Butyl hydroperoxide	1	i v	1 min	6
–	2	–	1 –	15
–	3	–	1 –	38
Ethyl linoleate	1	–	1 –	4
–	2	–	1 –	37
–	3	–	1 –	51
Triglyceride	1	–	1 –	0 6
–	2	–	1 –	0 6
Butyl hydroperoxide	8	i p	1 –	0
			15 –	33
			1 h	12
			2 –	5
Ethyl linoleate	8	–	1 min	0
			15 –	0
			1 h	0
			2 –	0
Triglyceride	2 5		20 –	0
–	5	–	20 –	0

constituents of the blood Butyl hydroperoxide is eliminated immediately after injection

The results given in table 2 show that a great part of the intravenously injected triglyceride peroxides can be recovered from certain organs shortly after the injection The main depositories are the lungs, then the liver Considerable quantities are found also in the spleen, kidneys and intestine and minor amounts in the heart and muscles In brain, adipose tissue and pancreas, only negligible quantities are found

When tween 80 is used as an emulsifier, relatively smaller quantities are recovered from the organs, especially from the lungs

Most injections were given into the jugular vein, but the lungs were found also to be the main depository after injection into the carotid artery

Considerable amounts of peroxide containing triglycerides were found in different organs and blood after 24 hours The amounts were smaller after 48 hours, but still significant After three weeks the peroxide had completely disappeared

When peroxides of substances other than triglycerides were injected, much smaller amounts were found in the organs after 1 hour (table 3) in particular, much less was recovered from the lungs When butyl hydroperoxide was injected, the recovery was nil

intravenous administration, but not when given intraperitoneally This substance can also probably enter the erythrocyte, although less readily than the smaller molecules of butyl hydroperoxide Part of it is deposited in the liver and elsewhere, but as the molecules are smaller and the substances are partly destroyed in the blood stream, a relatively smaller amount will be deposited than of the triglyceride peroxides

Ethyl linoleate hydroperoxide is also toxic, although less so than butyl hydroperoxide Even though, in the experiments reported in table 5, intraperitoneal administration did not produce methaemoglobin, in other experiments large amounts resulted in deaths The toxicity of intraperitoneal injections of linoleate peroxides has been studied by HORGAN, PHILPOT, PORTER & ROODYN (1957) and by HOLMAN & GREENBERG (1958) From the former's figures a LD50 in mice of about 0 5 μeq linoleic acid peroxide and 4–5 μeq methyl linoleate peroxide per g body weight can be calculated A LD50 of about 2 5 μeq ethyl linoleate peroxide per g body weight can be calculated from the figures of HOLMAN & GREENBERG Since no methaemoglobin is formed after intraperitoneal administration of ethyl linoleate peroxide, the mechanism of its toxic action must be different from that of butyl hydroperoxide Its toxicity is not explained by the inflammation produced in the abdominal cavity and elsewhere, since this was also produced by the less toxic triglyceride peroxides Possibly other essential enzymic or cellular systems are irreversibly oxidised or denatured

The experiments with smaller doses of peroxide-containing free fatty acids, phospholipids or cholesterol were not sufficient in number to permit definite conclusions about the fate of these three kinds of peroxides Probably most of them behave like ethyl linoleate hydroperoxide

Peroxide-containing triglycerides behaved in a strikingly different manner from ethyl linoleate and butyl hydroperoxides Large amounts were tolerated, and no evidence of methaemoglobin formation or other sign of specific toxicity was observed, even with the largest amounts used It seems as though the large molecules enter the erythrocytes slowly, but are quickly deposited in the reticulo endothelial system, and that this is able to metabolize large amounts The nature of the specific mechanism for the metabolism of peroxides is unknown Since a group of chicks *reared on a vitamin E-deficient diet* behaved in exactly the same way and gave the same peroxide recoveries as the control group and as chicks on normal diet, the tocopherols are probably not involved

The peroxide-destroying properties of liver have been studied by DUBOULOZ and his co workers (e g, DUBOULOZ, FONDARAI & LAGARDE 1949) Their comprehensive studies were carried out with liver brei, and

blood volume, 8 3% for chicks (PAPPENHEIMER & GRAFF 1932) and 6 7% for rats (GRIFFITH & FRANCIS 1942)

Even with the limitations mentioned above, the material presented in table 2 justifies the conclusion that the greater part of intravenously injected triglyceride peroxides disappears rapidly after elimination from the blood

In two experiments the animals were killed as soon as possible after the injection, time being allowed only for collecting a blood sample from the other jugular vein The organs were removed and placed on dry ice until the peroxide determinations could be carried out, in order to prevent post-mortem continuation of the peroxide destroying process In one of the experiments a large amount of peroxide was given (3 μeq /g body weight), about one third of the amount could not be accounted for In the second experiment (0 9 μeq /g) about 75% could not be recovered

It appears that, after the first phase of rapid elimination of peroxides the remainder disappears more slowly After 1 hour almost the same picture was observed as in the animals killed immediately after the injection, whereas after 24 hours only a small, and after 48 hours a still smaller amount was left

It appears from table 3 that other lipoperoxides disappear much more rapidly than peroxides of triglycerides A difference was also noted in the *toxic proertiesp* whereas even the largest amounts of peroxide containing triglycerides were well tolerated a dose of about 1 μeq /g body weight of butyl hydroperoxide was lethal, and deaths were also observed after the injection of ethyl linoleate hydroperoxide

The determination of the methaemoglobin levels of the blood of rats given different peroxides by intravenous or intraperitoneal administration showed a clear difference between peroxides of various origin (table 5) Intravenous butyl or linoleate hydroperoxides gave rise to rapid formation of large amounts of methaemoglobin, but significant amounts were not found after injection of peroxide containing triglycerides When the products were given intraperitoneally, only butyl hydroperoxide, but neither triglyceride nor ethyl linoleate peroxides produced methaemoglobinemia

The results support the hypothesis of a different behavior of different peroxides Butyl hydroperoxide possesses an acute toxicity which can be explained by its methaemoglobin forming potency This hydroperoxide apparently passes quickly through the erythrocyte membrane When methaemoglobin determinations were carried out, it was observed that the haemoglobins were only partly soluble in water Probably butyl hydroperoxide not only oxidises but further denatures haemoglobin

Ethyl linoleate hydroperoxide also produced methaemoglobin after

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it remains to be established whether or not the systems studied by DUBOULOZ were the same as those acting *in vivo*

No symptom of vitamin E-deficiency and especially no encephalomalacia was observed, after parenteral administration of peroxides Our studies, therefore, do not support theories that explain the manifestations of vitamin E-deficiency as a direct consequence of peroxide toxicity

NISHIDA, TSUCHIYAMA, INOUE & KUMMEROW (1960) produced encephalomalacia in chicks kept on a vitamin E-deficient diet by intravenous injection of methyl linoleate hydroperoxide The disorder developed in 1–5 hours after the injections, and the incidence and severity was greatly influenced by the diet The different behavior of different kinds of peroxides should be taken into consideration when these results are evaluated Our experiments indicate that the monoester hydroperoxide used in the experiments of NISHIDA and his co-workers was much more unstable and more toxic than the peroxide-containing triglycerides

The toxicity of lipoperoxides also has been credited with a decisive role in radiation toxicity (HORGAN, PHILPOT, PORTER & ROODYN 1957) In this connection it must be borne in mind that the peroxides, at least those of certain of the lipids occurring in larger amounts in the body, are much less toxic than the simpler peroxides

Summary.

Emulsions of various kinds of lipoperoxides were given parenterally to chicks and rats Large amounts of peroxide-containing triglycerides were well tolerated, even when given intravenously Ethyl linoleate hydroperoxide showed some toxicity, tertiary-butyl hydroperoxide was much more toxic

Methaemoglobinaemia occurred after administration of butyl hydroperoxide (i v or i p) and ethyl linoleate hydroperoxide (only i v) but after neither i v nor i p injection of triglyceride hydroperoxide

A large part of intravenously injected lipoperoxides disappeared rapidly However, considerable amounts of peroxide could be recovered from blood and various organs, especially lungs, liver, and spleen, up to 48 hours after i v administration of peroxide containing triglycerides Smaller amounts could be recovered, especially from liver, after i p administration Similar results, but relatively smaller recoveries, were obtained with peroxides of ethyl linoleate, phospholipids free fatty acids or cholesterol No recovery was obtained after giving butyl hydroperoxide

The results are discussed, and the different behaviour of different peroxides is stressed

Table 1

Effect of Ethanol on Noradrenaline Dopamine and 5 Hydroxytryptamine in Rabbit Brain.

Body weight kg	Ethanol		Time interval in hrs	Noradrenaline µg/g	Dopamine µg/g	5 HT µg/g
	dose g/kg	conc %(w/v)				
2 8	2	20	1	0 27	0 38	0 31
2 5	2	25	1	0 28	0 30	0 38
6 0	2	50	1	0 27	0 59	0 27
2 3	2	20	12	0 30	0 26	0 36
2 1	2	25	12	0 36	0 33	0 35
2 0	2	20	12	0 44	0 42	0 40
2 0	5	25	2	0 30	0 40	0 44
Average				0 32	0 38	0 36
Controls				*0 28*[1] ±0 017	*0 43*[1] ±0 016	*0 35*[2] ±0 014

[1]) Average of 5 determinations ± s e m
[2]) Average of 4 determinations ± s e m

cyanosis After about 30 minutes the animals began to recover slowly After one hour there was still some ataxia After 12 hours the animals seemed to be normal One rabbit, given 5 g/kg, was deeply anaesthetized even after two hours

In brain levels of noradrenaline, dopamine and 5 HT no difference was detectable between the normal and the ethanol treated rabbits (table 1) The normal values given in the table are in agreement with earlier results from this laboratory

Analogous results were obtained in the mice After the smaller dose the animals were markedly atactic, whereas animals given the larger dose were deeply anaesthetized No cyanosis was detectable Some recovery seemed to set in after about 90 minutes

After the smaller dose the brain levels of noradrenaline and 5-HT did not differ significantly from those of the controls (table 2) After the larger

Table 2

Effect of Ethanol on Noradrenaline and 5 Hydroxy tryptamine in Mouse Brain

Dose g/kg	Noradrenaline µg/g	5 HT µg/g
0	0 44[1]) ±0 021	0 53[2]) ±0 029
2 5	0 51	0 49
5 0	0 29	0 41

[1]) Average of 14 determinations (84 animals) ± s e m
[2]) Average of 8 determinations (48 animals) ± s e m

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From the Department of Pharmacology, University of Goteborg, Sweden

Ineffectiveness of Ethanol on Noradrenaline, Dopamine or 5-Hydroxytryptamine Levels in Brain

By

Jan Häggendal and Margit Lindqvist

(*Received July 19, 1961*)

Levels of noradrenaline and 5-hydroxytryptamine have been shown by GURSEY & OLSON (1960) to fall by about 50 per cent in the brain stems of rabbits after administration of ethanol This depression was parallel for the two neurohormones and persisted several days after ethanol had disappeared They found the effect of ethanol similar to that produced by reserpine and suggested that the hypnotic and sedative effects of the two drugs are caused by similar mechanisms

Attempts to reproduce these interesting experiments are described below

Methods.

Apparently healthy rabbits (2 to 6 kg) were slowly injected intravenously with ethanol, 2 g/kg body weight (absolute ethanol, "special for spectro scopy")

Various concentrations of ethanol in normal saline were used (table 1) The animals were killed by air embolism 1 or 12 hours after the infusion

Some experiments were performed on white mice One group of 6 mice received an intraperitoneal injection of ethanol 2 5 g/kg and another group received 5 g/kg The mice were killed 2 hours after the injection

The brains were immediately removed and homogenized with 0 4 N perchloric acid For the determinations the brains of the mice in each group were pooled The catechol amines were determined by the methods of BERTLER, CARLSSON & ROSENGREN (1958) and CARLSSON & WALDECK (1958) and 5 hydroxytryptamine (5 HT) by that of BERTLER (1961) The readings were taken in an Aminco-Bowman spectrophotofluorometer

Results.

During the infusion of ethanol, which lasted 5 to 10 minutes, the rabbits showed the usual signs of ethanol intoxication When laid down on their sides, they did not move They had nystagmus Conjunctival reflex was present Pain reactions were reduced There were no signs of

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From the Department of Forensic Medicine, University of Edinburgh

Toxicological Analysis by Direct Application of Biological Material to Ion-Exchange paper

By

Harold V. Street

(Received August 22, 1961)

Chromatography of untreated biological material applied directly to paper appears not to have been investigated for purposes of toxicological analysis There are probably two reasons for this First, the solvents generally employed in conventional chromatography would cause denaturation of the proteins in the biological material The resulting denatured protein would bind many compounds and thus prevent them from moving away from the protein on the chromatogram Secondly, the amount of toxic substance being sought is generally so small that often at least 2 ml of blood would have to be put on to the paper for the substance to be detected

During electrophoresis in aqueous solvents the proteins themselves are known to move under the influence of the electric field Hence, differentiation from other substances might be impossible or difficult GOLDBAUM (1960), however, has suggested that two-dimensional electrophoresis on "ordinary" filter paper is worthy of investigation He points out that by carrying out electrophoresis at a pH value at or near that of the isoelectric point of the proteins, it may be possible to cause the toxic agent to move in either one direction or the other, while the proteins remain at the point of application

SMITH (1960), however, states that deproteinisation of biological fluids is a necessary preliminary step in low-voltage (unmodified cellulose) paper-electrophoresis of amino acids It is my own experience, too, with low voltage electrophoresis on unmodified cellulose paper that, in general, deproteinisation is necessary when dealing with compounds of low molecular weight Further, EFRON (1960) states that in high-voltage paper-electrophoresis on (unmodified cellulose) paper, the presence of protein in biological fluids will cause streaking of substances of small molecular weight He therefore advises deproteinisation before examination

Using Whatman (DE20) *diethyl*aminoethyl-cellulose paper in 0 2N ammonia solution, I have found that, when either whole blood or serum

dose somewhat lower values were obtained In view of the fact that this comes fairly close to the lethal dose, the significance of the finding is doubtful

Discussion.

We are unable to give any explanation of the discrepancy between our results and those of GURSEY & OLSON It may possibly be due to differences in stock or diet Our results indicate, however, that ethanol can produce its typical actions without any change in the levels of noradrenaline, dopamine or 5-HT It thus does not seem very likely that ethanol and reserpine act by similar mechanisms, as suggested by GURSEY & OLSON

Summary.

An intravenous infusion of ethanol (2 to 5 g per kg) caused no significant change in the levels of noradrenaline, dopamine or 5-hydroxytryptamine in rabbit brain Analogous results were obtained in mice

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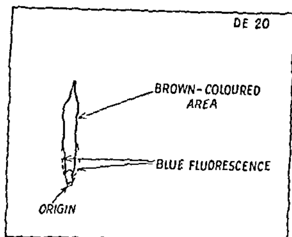


Figure 1 Direct application of blood from a case of salicylate poisoning 25 minutes chromatography on DE20 paper in 0 2 N–NH_3

is applied directly to the paper, the proteins do not migrate from the point of application either by chromatography or by ionophoresis A limiting factor here is the capacity of the paper, which can only handle a few microlitres of blood However, provided this is borne in mind, it is possible to effect a separation of certain substances from biological material applied directly to the modified cellulose paper Because only a small volume of protein-containing material can be applied to the paper such a technique is of value only when the concentration of toxic substance is relatively high

In order to illustrate the separation of drugs from blood proteins by direct application of the blood to the paper, two cases of suicide recently investigated in this Department will be described

The *first case* was one of death after ingestion of a large overdose of aspirin The blood was first analysed by the method of TRINDER (1954) and was found to contain 95 mg of salicylate per 100 ml A portion of this blood was taken up in a capillary tube and applied directly to a 4"×5" sheet of diethylaminoethyl cellulose paper Chromatography was carried out for 25 minutes in 0 2 N ammonia solution The pattern shown in fig 1 was seen when the wet paper was examined under the 254 mμ lamp The wet paper was then subjected to ionophoresis (5 milliamps constant current) in the same 'solvent' in a direction at right angles to the chromatography 'run' After 45 minutes, the paper was examined again The results are shown in fig 2 The characteristic blue fluorescent spot of salicylate had migrated towards the anode and was clearly differentiated from other absorbing material in ultraviolet light

The *second case* was one of death after an overdose of phenobarbitone Analysis of the blood by the method of CURRY (1961)

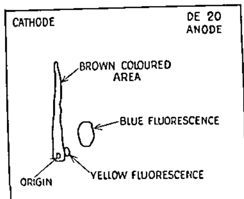


Figure 2 Direct application of blood from the same case of salicylate poisoning as used for figure 1 25 minutes chromatography and then 45 minutes ionophoresis at a constant current of 5 milliamps both procedures run in 0 2 N NH_3 The blue fluorescent spot is due to salicylate

contained 9 8 mg of barbiturate per 100 ml The barbiturate was identified as phenobarbitone by the method of STREET (1961)

The blood was then applied by a fine capillary tube to diethylaminoethyl cellulose paper Chromatography for 25 minutes in 0 2 N ammonia solution and then ionophoresis for 45 minutes at 5 milliamperes constant current (also with ammonia solution) in a direction at right angles to the first 'run' gave results shown in fig 3 A dark absorbing area was noted in the position that barbiturate had been previously shown to occupy (see STREET & NIYOGI 1961 a, 1961 b)

It is considered that this technique will be applicable to many other toxic substances, it should be particularly useful for analysis of gastric contents

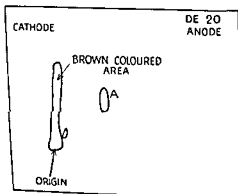


Figure 3 Direct application of blood from a case of barbiturate poisoning 25 minutes chromatography and then 45 minutes ionophoresis at a constant current of 5 milliamps both procedures 'run in 0 2 N-NH_3 A' = Phenobarbitone

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Retention of Selenium by Pig Tissues

By

C A Grant, B Thafvelin and R Christell

(Received August 10 1961)

With the announcement by SCHWARZ & FOLTZ in 1957 that simple selenium salts were effective in preventing dietetic liver necrosis produced under laboratory conditions in rats, biological interest in the element shifted from its toxic properties to its role, either as an essential dietary component or possibly through pharmacodynamic action, in suppressing certain lesions produced by dietary means

Dietetic liver necrosis in pigs (*hepatosis diaetetica* or HD in OBEL's, 1953, nomenclature) is also suppressed by dietary supplements of selenium salts (EGGERT *et al* 1957, GRANT & THAFVELIN 1958) So is dietetic microangiopathy in this species (MAP, see GRANT 1961)

Some claim that selenium is fully effective in preventing skeletal muscle degeneration (MD) in pigs (LANNEK *et al* 1960) but others have expressed doubt (GRANT & THAFVELIN 1958) "Yellow fat" – the deposition of acid fast pigment in adipose tissue – is not suppressed by selenium treatment of pigs (GRANT & THAFVELIN 1958)

MAP and HD, at least, are common and economically important pig diseases in Scandinavia Although not all the links in the chain from particular samples of feed to the final lesions in the pig are known at present there is good reason to suspect potential or actual oxidative instability of dietary fat, especially cereal fat, as the sine qua non (see THAFVELIN 1960 a, b) In that event the most rational therapy is dietary manipulation to ensure that ingested fats are stable

As an auxiliary measure, treatment of affected groups of pigs with selenium salts can be contemplated Selenium is highly toxic for mammals, and administration of the element or its salts to animals intended for human food can be justified only if it can be demonstrated that selenium retention in the tissues is minimal

Activation analysis as a means of determining selenium offers many advantages over chemical procedures and has been applied to the tissues of pigs used in experiments dealing with the induction of HD, MAP, and MD and the effect of dietary supplementation with sodium selenite. Some of these experiments have been described in previous papers

Methods

A. *Pigs.*

All pigs were of the Swedish lantras type. With the exception of those in Expt MII all pigs in a given experimental series were litter-mates Sow 11 was also the dam of the litter used in Expt D. Her 3 litters covered by this report were by the same boar.

B. *Feed.*

The pigs in Expts C, D, and F were fed the semi-synthetic soya meal I diet described by GRANT & THAFVELIN (1958) and in more detail by GRANT (1961) The crude protein content was about 20%

Fc [illegible] containing fat of low stabi [illegible] 960) The [illegible] skim milk is generally given as about 3 5%

The pigs in Expt MII received ground mixed grain with a protein content that can be assumed to be much the same as in the diet used in Expt E and, in addition, 0 25 l heated maize oil per pig per day

Selenium was administered to the appropriate groups as sodium selenite, (Na_2SeO_3), at 0 2 mg/kg dry feed except for sow *11* Some pigs received supplementary sodium selenite by intramuscular injection (approximately 0 01 mg selenium/kg body weight) Selenium supplementation was interrupted in some instances at various times before the animals were killed, during the intervening period the pigs were fed a standard low-protein ration Details are given in the tables

Sow 11 was given 0 4 mg Na_2SeO_3/kg dry feed, twice as much as was used in the other experiments Except during the immediate pre- and post-partum and suckling periods, her daily ration consisted mainly of ground grain and potatoes Selenite supplementation was maintained throughout the greater part of 3 successive pregnancies and was begun on the thirty-seventh day of the first *She was slaughtered* 425 days later No selenite was given during the 30 days before slaughter or for *periods of one week before and after the first two parturitions*, the third litter was born 3 weeks before slaughter The number of "Se days" for this sow was accordingly 367

Except *for sow 11*, individual feed consumption was impossible to assess The sow was fed individually but the quantity of feed was varied as her condition demanded

C. *Tissues.*

At the time of death or slaughter, one kidney and samples of liver and skeletal muscle (*M. semimembranosus*) were removed and stored at $-20°C$ until activation analysis could [illegible]

from each sample, part of each slice was sealed in a quartz ampoule for neutron irradiation and part was dried at 105° for 12 hours for determination of dry matter content and then ashed

D *Activation Analysis*

Neutron irradiation of selenium gives Se^{81m} with a half life of 57 minutes and Se^{75} with a half life of 121 days

Se^{81m} is formed from Se^{80} which comprises 49 8% of natural selenium and Se^{75} from Se^{74} 0 87% of natural selenium Se^{81m} decays by isomeric transition with the emission of γ radiation to Se^{81} and then by β-radiation to Br^{81} Se^{75} decays by electron capture and γ radiation to As^{75} (KINSMAN 1957 ALLEN *et al* 1959) Spectrometric activation analysis involving the γ radiation of Se^{75}, alone or in combination with chemical separation was applied to the pig tissues

For *spectrometric activation analysis* tissue samples of approximately 1 g were sealed in quartz ampoules and neutron irradiated together with a Se standard in the R1 reactor Stockholm for approximately 5 days at a flux of $1\ 5 \times 10^{12}$ neutrons per cm^2 sec After aging for at least 2 months, the γ spectra (Se^{75}) of the samples were measured in a single channel pulse height analyser coupled with a scintillation detector with a 3 × 3 NaI (Tl) crystal The ratio between the areas under the photo-electric peaks at γ-energies of 121 + 136 and 265 + 280 keV (double peaks) for sample and standard is identical with the ratio between the Se contents of the sample and the standard

For *chemical separation* after activation analysis a carrier of inactive selenium was added to the irradiated sample selenium was separated chemically and total activity was measured with a scintillation counter fitted with a well type NaI crystal Through γ ray spectrometry it could be shown that selenium was the only active element present Since the amount of selenium added was known the chemical recovery could be calculated (nearly complete) For solution the method described by MONIER-WILLIAMS (1949) was utilised and for distillation and precipitation that of FINEMAN *et al* (1959)

Through the following procedure selenium was separated from the pig tissues sealed in quartz ampoules

1) Place approximately 70 mg HgO in a 500 ml distillation flask

2) Crush the quartz ampoule in a plastic tube and rinse out the tube with a chilled mixture of 10 ml H_2SO_4 and enough HNO_3 to correspond to 10 ml/g sample

3) Add as carrier a known amount (approximately 70 mg) Na_2SeO_3.

4) Connect a refl [illegible]

gas

[illegible]

8) Continue distillation until the temperature reaches 130

9) [illegible]

13) Weigh the precipitate after drying at 105°.

14) Dissolve the precipitate in approximately 2.5 ml HBr–Br_2 solution and allow to drop into a small polyethene test-tube.

15) Add approximately 2 5 ml H_2O as diluent.

16) Transfer the tube containing the final solution to a well-type scintillation crystal, and measure the activity

Results

The retention of Se in kidney, liver, and skeletal muscle tissue (*M. semimembranosus*) expressed as p.p.m. (mg/kg) for the wet tissue is given in tables 1 to 5 for pigs of Expts C, D, E, F, and MII and in table 6 for sow 11 and 2 of her litters. Details of the duration of Se feeding, supplementary injections of Se if any and Se-free periods before slaughter are

Table 1.

Exp't F. Selenium retention in pig tissues after various periods on Soya I diet with or or without addition of 0 2 mg Na_2SeO_3/kg Spectrometric activation analysis, Se as p p m wet tissue The vaules given for γ-spectrometry represent the calculated Se content and limits of error

Pig	Se days	Supplementary Se	Se free days	Skeletal muscle	Liver	Kidney
With selenite						
40 F	100	–	–	<0 3	<0 3	1 5±0 2
42 F	100	–	–	0 6±0 2	0 3±0 2	2 1±0 3
41 F	101	–	–	<0 3	<0 3	1 1±0 2
43 F	101	–	–	<0 4	<0 3	1 8±0 3
44 F	101	–	–	<0 3	<0 4	1 4±0 2
Without selenite These two pigs died on the 44th day of the experiment						
45 F	–	–		<0 3	<0 4	0 6±0 2
49 F	–	–		0 3+0 2	0 4±0 2	0 5±0 2

Table 2.

Expt' D Selenium retention in pig tissues after various periods on soya I diet with addition of 0 2 mg Na_2SeO_3/kg Spectrometric activation analysis, Se as p p m wet tissue

Pig	Se days	Supplementary Se	Se-free days	Skeletal muscle	Liver	Kidney
Se prophylaxis						
20 D	95	3 × 0 3 mg	–	0 4 0 2	0 4±0 2	1 6 0 3
21 D	97	3 × 0 3 mg	–	<0 4	<0 4	2 5±0 4
24 D	98	3 × 0 3 mg	–	1 0±0 2	0 4±0 2	1 9±0 3
25 D	99	–	–	<0 4	1 0±0 2	1 9±0 3
Se therapy						
27 D	42	4 × 0 5 mg	–	0 6±0 2	0 7±0 2	1 5±0 3
22 D	45	4 × 0 5 mg	–			
28 D	57	5 × 0 5 mg	–			

Table 3.

Exp t M II Selenium retention in pig tissues after various periods on a grain and vegetable oil diet with or without addition of 0.2 mg Na_2SeO_3/kg grain Spectrometric activation analysis, Se as p p m wet tissue

Pig	Se days	Supplementary Se	Se free days	Skeletal muscle	Liver	Kidney
With selenite						
65	70	–		<0.4	0.6±0.2	1.3±0.2
66	70	–	–	<0.4	0.8±0.2	1.8±0.3
67	70	–	–	<0.3	0.8±0.2	1.8±0.3
68	70	–	–	<0.3	0.3±0.2	2.0±0.3
Without selenite Killed on the 72 nd day of the experiment						
70	–	–		0.3±0.2	0.6±0.2	1.0±0.2
71		–		<0.4	<0.4	0.8±0.2

Table 4

Exp t C. Selenium retention after various periods on Soya I diet with addition of 0.2 mg Na_2SeO_3/kg and subsequent periods on a standard ration without added Se Spectrometric activation analysis, Se as p p m wet tissue

Pig	Se days	Supplementary Se	Se free days	Skeletal muscle	Liver	Kidney
6 C	13	1 × 0.7 mg	22	0.5±0.2	–	2.0±0.3
4 C	22	3 × 0.5 mg	10	<0.4	–	2.2±0.3
1 C	33	4 × 0.25 mg	22	0.6±0.2	–	2.0±0.3
2 C	33	4 × 0.5 mg	22	0.3±0.2	–	1.4±0.3
3 C	33	4 × 0.6 mg	22	0.4±0.2	0.4±0.2	2.5±0.4
7 C	33	4 × 0.8 mg	22	<0.4	–	2.5±0.4
8 C	33	3 × 0.55 mg	22	<0.4	–	1.9±0.3

Table 5.

Exp t E Selenium retention after various periods on grain ration with or without addition of 0.2 mg Na_2SeO_3/kg and subsequent withdrawal of selenite for various periods Spectrometric activation analysis, Se as p p m wet tissue

Pig	Se days	Supplementary Se	Se free days	Skeletal muscle	Liver	Kidney
With selenite						
32 E	100	–	11	<0.3	0.3±0.2	1.6±0.3
33 E	100	–	11	<0.3	0.5±0.2	2.4±0.3
30 E	100		47	<0.5	0.7±0.2	2.1±0.3
31 E	100		47	<0.5	0.9±0.2	1.7±0.3
Without selenite						
37 E[1]				<0.3	<0.3	0.5±0.1
34 E	–			0.5±0.2	0.4±0.2	1.2±0.3
35 E	–			0.3±0.2	<0.4	1.8±0.3
36 E	–	–		<0.4	0.4±0.2	1.4±0.3

[1])Died after 59 days The other pigs in the untreated group were killed after 111 days and those in the selenite group after 111 and 147 days not 8 days later as incorrectly stated 60)

Table 6.

Selenium retention in sow 11 (0 4 mg Na_2SO_3/kg feed) and in two of her litters Spectrometric activation analysis, *Se as p p m wet tissue*

Pig	Se days	Se free days	Skeletal muscle	Liver	Kidney
Sow 11	367	30	0 3±0 2	0 8±0 3	2 6±0 5
	Age at death, days				
Se litter I					
0 6495/58	1		<0 3	0 8±0 2	0.5±0 2
0 6496/58	1		<0 3	0 6±0 2	0 6±0 3
0 6625/58	6		0 4±0 2	0 5±0 2	0 6±0 2
0 6732/58	11		<0 3	1 0±0 2	0 7±0 2
0 6772/58	13		<0 3	0 5±0 2	0 6±0 2
Se litter II					
0 2799/59	1		<0 3	0 8 ±0 2	0 7±0 2
0 2800/59	1		<0 4	1 1 ±0 2	0 8±0 2

also included in the tables Farrowing data for sow 11 are given in table 7

Results obtained by γ-spectrometry alone and total caunting after chemical separation are compared in table 8

The main pattern of selenium retention was fairly uniform in all the experiments Except for the piglets of sow 11, selenium retention was greatest in the kidneys Values obtained for skeletal muscle and liver were of a lower but much the same magnitude

Some of the animals in these experiments were not given selenium orally or parentally (see tables 1, 3 and 5) The selenium content of

Table 7

Parturition data and litter size (live piglets) for sow 11

Litter size	Gestation days	Parturition interval days	Comment
7	114		
11	114	171	
11	114	151	
10	120	149	
11	114	136	
			Na_2SeO_3 0 4 mg/kg feed
17	114	153	Se litter I
14	118	175	Se litter II
5	114	152	
10 8	115 4	Mean	

Table 8

Activation analysis for Se in pig tissues Comparison of results obtained by γ-spectrometry of irradiated samples and by total caunting after chemical separation Se, p p m in wet tissue

Pig	Skeletal muscle		Liver		Kidney	
	γ spectr	chem	γ spectr	chem	γ spectr	chem
40 F			<0 3	0 23±0 02		
41 F					1 1±0 2	1 27±0 05
42 F			0 3±0 2	0 21±0 2		
43 F	<0 4	0 11±0 02	<0 3	0 41±0 02	1 8±0 3	2 03±0 08
44 F					1 4±0 2	1 52±0 06
45 F	<0 3	0 07+0 01	<0 4	0 08±0 01	0 6±0 2	0 48±0 02
49 F	0 3+0 2	0 05±0 01			0 5±0 2	0 60±0 02
34 E	0 5±0 2	0 45±0 02	0 4±0 2	0 15±0 02	1 2+0 3	1 24±0 05
35 E	0 3+0 2	0 09+0 02	<0 4	0 26±0 02	1 8 0 3	1 43±0 06
36 E	<0 4	0 07±0 02	0 4+0 2	0 34±0 02	1 4±0 3	1 25±0 05
37 E	<0 3	0 10±0 02	<0 3	0 20±0 02	0 5+0 1	0 85±0 04

muscle and liver tissue in these pigs fluctuated around the same lower limits as in the pigs given sodium selenite Except in Expt E (table 5) the amount of selenium in the kidneys was slightly greater than in muscle or liver but less on the whole than in the kidneys of the pigs of the selenium group In Expt E, apart from pig 37E, which died of dietetic microangiopathy, *hepatosis diaetetica*, and waxy degeneration of the skeletal muscles, the non selenite pigs had relatively appreciable amounts of selenium in their kidneys Confirmation of this incongruously high selenium content can be seen in table 8 The grain fed in this experiment may have contained a relatively large amount of selenium, no sample is now available for selenium determination

For the piglets of sow 11, the selenium content of skeletal muscle was somewhat less than that of the other tissues, but there was no apparent evidence for selective accumulation in the liver or kidneys

All the pigs in the selenite groups (except sow 11) were given the same relative amounts of sodium selenite, 0 2 mg/kg feed Over the whole course of the experiments individual feed consumption is unlikely to have varied much judging from the weight gains The amo
sumed each day can l
ning to the end of th
expected to eat about 2 kg solid feed per day This would give a daily intake of 0 4 mg Na_2SeO_3 or 0 01 mg/kg body weight

The remarkable uniformity in the pattern of results for the selenite

pigs in tables 1 to 5 and for sow 11 in table 6 was not noticeably affected by the duration of Se feeding (compare tables 1 and 4) or the length of the Se-free period before slaughter (table 5 for example) The relatively small amounts of Se (approx 0 01 mg/kg body weight) given to some of the pigs by intramuscular injection in the neck did not have a discernible effect on Se retention

Chemical separation before activity measurements gave greater accuracy at the cost of much more labour The advantages of the combined analysis are especially apparent for tissues containing little selenium (table 8)

Comment

There has been no opportunity to check the results obtained here by γ-spectrometry with other methods than chemical separation of selenium before counting total activity (table 8) As a selenium carrier was added and recovery was calculated for each sample, the maximum error for this phase can be estimated as not greater than $\pm 2\%$ Measurements of radioactivity could be carried out with a maximum error of $\pm 3\%$ The combined error for chemical separation and γ-activity measurements did not exceed $\pm 5\%$

For the samples analysed solely by γ-spectrometry after neutron irradiation, measurements of the area under the photo peak at 121–136 keV (the largest) could be carried out with a precision of ± 1 to $\pm 10\%$ The appearance of the spectrum gave an estimate of the slope of the background line under the selenium peaks If relatively large amounts of selenium were present, the peaks were distinct, and the slope of the background line could be readily estimated If the peaks coincided to a great extent with the background line, so that a distinct rise was not produced, the amount of selenium present was considered to be at the limits of detection This limit was approximately 0 2 μg Measurement of the areas of peaks of this magnitude naturally involved a large error

A comparison of the double peaks at 121–136 and at 265–280 keV made possible evaluation of the precision

A standard was always irradiated together with the samples The possibility has been considered that the induced activity in the standard and the samples might differ because of slight differences in position within the reactor No appreciable error due to this factor could be demonstrated

The use of activation analysis for selenium in animal tissue in the concentration range 0 001–10 0 ppm has been reported by LEDDICOTTE *et al* (1958) but they did not describe the method in detail Other methods

for determining selenium –colorimetric (RAY 1959) and fluorometric (WATKINSON 1960)– are capable of detecting it in trace amounts One of the advantages of activation analysis, however, is its freedom from the source of error introduced by selenium contamination of chemical reagents

The distribution pattern obtained for selenium in the various tissues is unlikely to have been affected much by variations in dry matter or ash content The dry matter content of the liver samples ranged from 25 to 30%, of kidney from 17 to 26% and of muscle from 20 to 29% Corresponding ash contents were 1 2 to 1 6%, 1 1 to 1 4% and 1 1 to 1 3%, respectively There was no apparent relationship between the Se and dry matter contents of individual samples

The tenacious retention in the kidneys of selenium fed to pigs in micro-amounts (0 2 mg Na_2SeO_3/kg feed) is compatible with some but not all of the results previously reported for selenium accumulation or retention in animals of various species (MCCONNELL 1941, DUDLEY 1936, MILLER & SCHOENING 1938, SMITH *et al* 1937, 1938, MOXON 1937, ROSENFELD & BEATH 1945)

Feeding rats on wheat containing 15 ppm Se resulted in accumulation of Se in the tissues reaching its peak after 4 to 8 weeks Withdrawal of selenium resulted in practically complete excretion of Se within 12 weeks (MUNSELL *et al* 1936)

ANDERSON & MOXON (1941) fed rats on seleniferous wheat (18 ppm Se) and found that 63 5% of the ingested Se was retained in the tissues during the first week There was a steady decrease in relative retention to 40% by the end of 7 weeks Young rats retained more Se than did older

In a similar experiment on rats, but with much greater amounts of Se as selenite (25 to 50 ppm), GORTNER & LEWIS (1939) recovered only 0 4 to 2 8% of the ingested Se in pooled samples of kidney, liver or spleen from rats receiving selenite for 28 to 143 days

Selenium accumulation was considerably greater from ingested naturally-occurring organic Se compounds than from similar amounts of Se as inorganic Se (SMITH *et al* 1938) Renal excretion of Se fed as inorganic Se rapidly declined after administration ceased If, however, Se was given as an organic compound renal excretion declined much more gradually (SMITH *et al* 1937, 1938, WESTFALL & SMITH 1940)

The rate of excretion when Se was given as a single dose of an inorganic compound has been reported as fairly rapid For rats HARSCHFIELD (1953) found total recoverable Se to be about 75% of the dose 5 hours after injection and about 65% after 12 hours, with no further decrease up to 48 h[illegible]

of at

and faecal excretion of Se by mice to be about 45% within the first 24 hours The mice of HEINRICH & KELSEY (1955) retained only 16 5% of the dose after 48 hours

Apart from urinary and faecal excretion, there is another pathway for excretion, namely, the expired air SCHULTZ & LEWIS (1940) claimed that between 17 and 52% of Se injected into rats as Na_2SeO_3 was excreted in expired air within 8 hours McCONNELL's (1942) results indicated that only 3 to 10% of the dose was expired in 24 hours

BLINCOE's (1960) paper on whole-body turnover of Se by the rat is of particular interest From his measurements of whole body radiation after the injection of Se^{75} as selenite, he was able to establish two constants for whole body turnover His conclusion from this was that Se is changed from a rapidly excreted into a slowly excreted form Processes limiting the excretion rate are either different renal clearances for two chemically different forms of Se or the existence of a slower excretion constant which reflects the rate of release of Se from tissues to which it is fixed

Selenium fed at 10 ppm, the only level tested, has been shown to reduce the fertility of sows, to increase the number of piglets dead at birth and to reduce the viability of surviving piglets (WAHLSTROM & OLSON 1959) The much smaller amount of selenium fed sow 11 in this experiment did not obviously affect her fertility, and she conceived at the first service The low number of piglets in her last litter is not unexpected in a sow that had farrowed 8 times in 4 years

Little is known of the fate of ingested Se in the mammalian body other than that it can become incorporated in compounds that normally contain sulphur, which it replaces (see for example McCONNELL & WABNITZ 1957, McCONNELL & KREAMER 1960) The presence of Se in SCHWARZ's Factor 3 has already been mentioned, in spite of intensive research, particularly by SCHWARZ and his group, the precise chemical structure and metabolic activity of Factor 3 are unknown, but it can only be a question of time before these are established

The purpose of our study has been to ascertain the amount of selenium retained in the tissues of pigs given enough sodium selenite to suppress the occurrence of certain dietetic lesions Perhaps the amount of selenite administered was excessive, only further research can decide ORSTADIUS & ÅBERG (1961) gave 7 pigs a single dose of Se^{75} as selenite with or without carrier Se by different routes Calculations of tissue retention were made by measuring only the amount of tissue radioactivity at intervals after dosing, not by measuring the amount of Se^{75} plus carrier Se in fact present By extrapolation they concluded that the amount of selenium retained in the tissues a week or more after this single dose would be unlikely to constitute a health hazard to human being who happened to

eat treated pigs Their calculations were based on the claim by LANNEK *et al* (1961) that a parenteral dose of 0 06 mg Se/kg body wt over 3 days would cure muscular degeneration in pigs We cannot subscribe to this embracing view on the basic of ouer findings (GRANT & THAFVELIN 1958, GRANT 1961)

The amount of tissue selenium demonstrated in our pigs, particularly in the kidneys, cannot be considered unduly great and there are few people who subsist mainly upon pig kidneys, yet the risk of poisoning human beings, to say nothing of pigs, through carelessness or accident would remain if selenite supplementation of pig feed were to become general practice

None of our pigs showed the lesions usually associated with selenium toxicity Degeneration of the lumbar-sacral intervertebral disc, however, was noticed in a few animals This observation, knowledge of the active sulphur metabolism in parts of the intervertebral discs (HANSEN & ULLBERG 1960), and COHR's (1949) mention of disc degeneration in connection with selenium poisoning of foals prompted us to look further into this question We (see HANSEN 1959 a) found that the lumbar-sacral disc has degenerated in about 5% of apparently normal slaughter pigs (about 6 months old) Preliminary experiments on mice maintained over a year on diets similar to those fed to our pigs and containing various amounts of selenite failed to provide definite evidence for a relationship between diet, selenium and disc degeneration (*see* HANSEN 1959 b)

It is difficult to say from the results of toxicity experiments, particularly chronic toxicity studies, just how much selenium can be tolerated in the diet by human beings or animals The form in which selenium is ingested, the species (and the wide individual susceptibilities), the composition of the diet and the particular criteria applied all contribute towards the lack of certainty (see TRELEASE & BEATH 1949) The lower figure of about 2 mg Se kg diet given by MUNSELL *et al* (1936) has been more or less tacitly accepted as the minimum toxic amount for prolonged ingestion

Nor is it clear whether or not human beings who live in seleniferous areas of the world and who therefore probably consume relatively large amounts of selenium suffer any ill effects There are only a few reports on this subject SMITH (1941), *in summarising the field surveys carried* out by himself and his collaborators in the western United States, concluded that they had been unable to find evidence for selenium toxicity in samples of the rural population, but the 'incidence of vague symptoms of ill health, particularly those suggestive of gastric and hepatic disorders, appeared to be sufficiently high to indicate the probability of cause and effect'

Fortunately, the widespread and fatal pig diseases against which *selenium is fully effective* – dietetic microangiopathy and hepatosis diaetetica – can be controlled by other methods Now that the properties of feed grains and their fats responsible for these diseases are being worked *out and practical prophylactic methods* are being evolved (THAFVELIN 1960 a, b) the use of selenium salts need be considered only as an emergency and auxiliary solution

Summary

Selenium retention in various tissues of pigs maintained on diets supplemented with sodium selenite (0 2 mg/kg) was ascertained by activation analysis (activity measurements after neutron irradiation) Kidney tissue retained more selenium than liver or muscle tissue In these experiments the distribution patterns and absolute amounts of retained selenium were not consistently affected by the duration of selenite supplementation (from 13 to 367 days) or the length of the selenium free period before slaughter and collection of tissues (from 0 to 47 days)

Although the amount of selenium retained in the pig tissues at the dose levels tested was relatively small (from 1 1 to 2 6 mg/kg kidney) there is a risk of poisoning occurring through indiscriminant and *careless supplementation of pig feeds Since the dietetic lesions of pigs* against which selenium is fully effective can be controlled by other methods, the use of selenium salts can be relegated to the position of an *auxiliary and emergency measure*

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From the Biological Department, The Pharmaceutical Institute,
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Reversal of the Inhibitory Action of Adrenaline and Histamine on Rat Uterus

By

Kjell Briseid Jensen and Anne Marie Vennerød

(Received June 23, 1961)

Results of quantitative experiments on the inhibitory action of adrenaline on the rat uterus have been published previously (BRISEID JENSEN & SUND 1960) With the object of comparing the mode of action of adrenaline with that of histamine, we also attempted to block the adrenaline effect with dichlorophenyl-2-isopropylaminoethanol (DCI) This substance, however, which is supposed to be a relatively specific blocking agent for the inhibitory action of adrenaline on various smooth muscles, itself depresses the rat uterus rather markedly (POWELL & SLATER 1958) Hoping to adjust the relative amounts of DCI and adrenaline in such a way that a decrease in the adrenaline effect could nevertheless be demonstrated, we conducted some experiments with various concentrations of the substances In no instance, however, could the adrenaline inhibition for certain be said to be reduced (BRISEID JENSEN & SUND 1960)

In the experiments reported here, by altering to some extent our experimental technique and by carrying out a considerable number of experiments, we were sometimes able to demonstrate a reversal of the adrenaline effect

Histamine was also tested and was found sometimes to stimulate the muscle The histamine reversal, however, did not require the use of DCI

Dibenzyline and mepyramine were used to test the specificity of the stimulating effects observed

1. Technique.

A. *Procedure*

Uterus and dose cycle Uteri from virgin rats weighing 150–180 g were suspended in de Jalon solution (GADDUM, PEART & VOGT 1949), usually maintained at 29–30° (±0 1°) The rats were injected with stilboestrol (10 µg/100 g) 18–24 hours before they

were killed The uterus was generally used soon after death, but was occasionally stored at +4° for not more than three days before use

The muscle was attached to an isotonic frontal writing lever (SCHILD 1947) yielding a magnification of about 5 The loads ranged from 0 5–0 9 g for human urinary kinin, and from 0 8–1 1 g for serotonin (5-hydroxytryptamine) The periods of contact with the agonist solution ranged from 45–80 seconds for human urinary kinin, from 30–75 seconds for serotonin The interval between doses was fixed at 4 minutes The antagonist solutions were put into the bath about 90 seconds before they were replaced by the agonist solutions The bath was supplied with air

Agonist and antagonist solutions Serotonin at concentrations of 3–20 ng/ml or human urinary kinin at concentrations of 15–40 mU/ml were used as agonists

The adrenaline, isoprenaline and noradrenaline solutions were freshly made every 45 minutes from stock solutions (25 mg base per litre) prepared with sufficient hydrochloric acid to give a pH of about 3 For dilution we used de Jalon solution containing 10 mg ascorbic acid per litre The solutions were protected against light throughout the experimental period

Apparatus The experiments were conducted in an automatic assay apparatus from the Casella Electronic Ltd London The bath volume was 2 ml, and the fluid was changed by emptying the bath and refilling it from stock bottles containing de Jalon solution, de Jalon solution with antagonist or de Jalon solution with agonist The washing between agonist doses consisted of a threefold change of the bath fluid

B *Materials*

Adrenal[illegible]

[illegible] yline ®) Smith,

Kl[illegible] Laborat[illegible]ories, Philadelphia

1 Dichlorophenyl 2 isopropylaminoethanol (*DCI*) The hydrochloride salt of the drug was kindly furnished by Eli Lilly & Co , Indianapolis

Histamine, Histamine di HCl, Light & Co , Ltd , Colnbrook

Human urinary kinin, A concentrate of the rat uterus stimulating principle in urine, prepared by the method of BRISEID JENSEN & SUND (1959)

Isoprenaline Isoprenalini sulfas Siegfried A G Sofingen

Mepyramine Mepyramini maleas, Delta Chemical Works, Inc , New York.

Papaverine Papaverin hydrochloricum, C H Boehringer Sohn, Nieder Ingelheim a Rhein

Serotonin 5-hydroxytryptamine creatinine sulphate, Hoffmann la Roche & Co , Ltd Basel

Noradrenaline L-Noradrenaline bitartrate, Light & Co , Ltd , Colnbrook

The doses of dibenzyline, DCI, isoprenaline, papaverine and serotonin mentioned in this paper refer to the salts used The adrenaline histam[illegible]

[illegible]

2. Experimental and Results.

A *Adrenaline Reversal after DCI*

As soon as the muscle had become stable on a submaximal dose of the agonist, an antagonist dose of adrenaline was usually given to check that

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to contract rat uterus, whereas isoprenaline did not The action of these two drugs, however, was only tested in a few experiments

B *Histamine Reversal after DCI*

The effect of histamine was examined in the same way as that of adrenaline, and it also was found occasionally to stimulate rat uterus (in 13 out of 28 experiments) Even if histamine often stimulated a muscle preparation that could also be stimulated by adrenaline, this did not always happen sometimes only histamine had a reversing effect, some times only adrenaline The doses of histamine used were of the order 1-20 µg/ml The contractions occurred much later than those after adrenaline They did not always end during the 90 seconds elapsing between addition of antagonist and addition of agonist Contrary to the way in which adrenaline stimulated by producing a real contraction, histamine sometimes only stimulated indirectly by considerably increasing the contractions of the 2–3 following agonist doses Histamine often caused both a direct and an indirect stimulation (fig 1)

C *Histamine Reversal without DCI*

BONNEY & FERGUSON (1950) describe experiments in which adrenaline had a biphasic effect on the rat uterus, particularly from rats in oestrus In order to discover whether we could also demonstrate an adrenaline-induced stimulation of the rat uterus without DCI under our experimental conditions we conducted several experiments in which a normal inhibitory dose of adrenaline or histamine was given at the beginning of the experiment and after several hours In no instance could a reversing effect of adrenaline be demonstrated, even during prolonged experiments (up to 12 hours with serotonin stimulation every fourth minute) Neither have we observed a biphasic or a stimulatory effect of adrenaline in previous experiments on adrenaline inhibition (BRISEID JENSEN & VENNEROD 1961 a b) Histamine however, often reversed its inhibitory effect in the course of the experiments Such a change could be observed when the uterus had been regularly stimulated (usually with serotonin) for some hours without any antagonist, as well as when the usual inhibitory dose of histamine (1–3 µg/ml) had been given in every cycle When the last-mentioned procedure was used, a gradual change from inhibition to stimulation could be demonstrated (fig 2) If adrenaline or papaverine was then tested, both inhibited as usual Sometimes only an indirect histamine stimulation was obtained, sometimes also real contractions The histamine-induced stimulation was reversed by mepyramine, as is also reported below for stimulation after DCI This effect of mepyramine

it was inhibiting the contractions normally An adrenaline concentration of 0 1–1 0 ng/ml normally reduced the contraction height by about 50% After a few recovery cycles without antagonist, DCI was given at a concentration inhibiting the contractions by 30–60% (usually 5 10 ng/ml) When a relatively stable inhibition plateau had been obtained the DCI solution was replaced by wash fluid (de Jalon solution) for some cycles, and adrenaline was then given as antagonist at a fairly high concentration – 50–150 ng/ml During the "washing out" of the DCI the contraction height often rose a little, but flattened out at a level demonstrating clearly that the drug had not in fact been removed As previously stated (BRISEID JENSEN & SUND 1960), DCI cannot be eliminated even by prolonged washing

By the procedure described an immediate contraction was caused by the added adrenaline in several experiments (9 out of 30) After the contraction, the first agonist dose, and sometimes also the second was completely inhibited (fig 1) When a normal contraction level had been re established after some cycles without DCI, a further addition of adrenaline reproduced the contraction, however, sometimes only one adrenaline contraction could be obtained Noradrenaline was also found

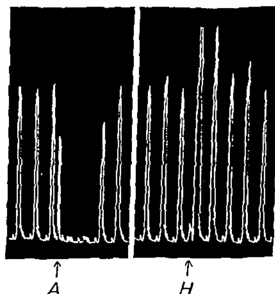


Fig 1 Adrenaline and histamine reversal in DCI treated rat uterus
Agonist Human urinary kinin 20 mU/ml
Adrenaline (A) concentration 90 ng/ml
Histamine (H) concentration 20 µg/ml
Uterus pre treated with DCI 5 ng/ml for 12 cycles and washed for 16 cycles before the adrenaline addition
12 minutes = 3 cycles between the two parts of the tracing

what might be expected, since dibenzyline is known to disappear only slowly from the muscle In a control experiment it was demonstrated that mepyramine did not block the adrenaline induced contractions (fig 4) The figure also shows the transitory increase in contraction height after adrenaline inhibition, which has also been previously described (BRISEID JENSEN & SUND 1960)

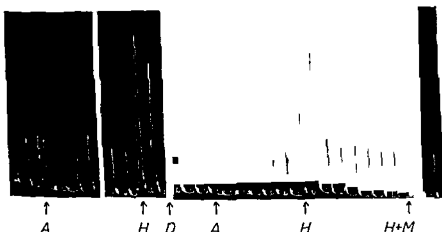


Fig 3 Adrenaline and histamine reversal in DCI treated rat uterus
Specific blockade by dibenzyline and mepyramine
Agonist Human urinary kinin 15 mU/ml
Adrenaline (A) concentration 62 5 ng/ml
Histamine (H) concentration 20 µg/ml
Dibenzyline (D) concentration 0 25 µg/ml left in the bath for 8 minutes
Mepyramine (M) concentration 10 ng/ml

Uterus pre treated with DCI 5 ng/ml for 17 cycles and washed for 11 cycles before the adrenaline addition

Time intervals between the 3 separate cuts of the tracing 28 and 40 minutes

3. Comments on the Experimental.

A *Adrenaline and Histamine Reversal after DCI*

In previous DCI adrenaline experiments (BRISEID JENSEN & S...

... which, in the first type of experiment, adrenaline was applied as antago...

... In some experiments no change in

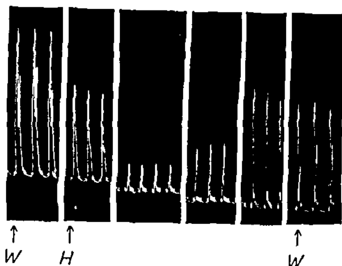


Fig 2 Time induced histamine reversal in rat uterus

Agonist Serotonin, 20 ng/ml
Histamine (H) given as antagonist (3 µg/ml) in the marked and the subsequent cycles
Washing fluid (W) without antagonist given in the marked and the subsequent cycles
At the first W the uterus had been stimulated for 88 minutes
Time intervals between the 6 separate cuts of the tracing 32 112 172, 192 and 4 minutes

seemed long lasting In an experiment in which histamine stimulation was first reproduced several times in the course of two hours, we could demonstrate histamine inhibition after mepyramine, reproducible at least over a similar time, in spite of continuous serotonin stimulation and washing

D *Specific Blockade of the Reversal Effects after DCI*

In experiments in which a reproducible stimulating effect of adrenaline could be demonstrated, dibenzyline (0 25 µg/ml) was left in the bath for 8 minutes This procedure did not significantly inhibit the muscle when human urinary kinin was used as agonist The stimulating effect of adrenaline, however, was abolished In analogous experiments with histamine as stimulating agent, the combined use of histamine and mepyramine eliminated the histamine-induced contractions In an experiment in which a reproducible stimulating effect of *both* adrenaline (62 5 ng/ml) and histamine (20 µg/ml) was obtained, the use of dibenzyline (0 25 µg/ml for 8 minutes) reversed the adrenaline effect, whereas histamine still stimulated strongly When a mixture of histamine (20 µg/ml) and mepyramine (10 ng/ml) was then given as antagonist, a complete blockade of the histamine stimulation was obtained (fig 3) When the contraction level in the course of some cycles without antagonist was re established, both drugs inhibited normally For adrenaline this was

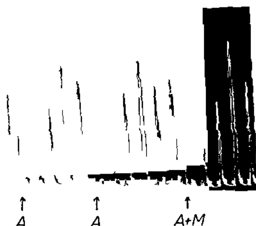


Fig 4 Adrenaline reversal in DCI treated rat uterus

zyline ® abolished the stimulating effect suggests that the mode of adrenaline action on rat uterus may be qualitatively similar to the mode of action on the smooth muscles of vessels probably also on other types of smooth muscles There are effects both on structures leading to an activation of the contractile elements and on structures whose stimulation inhibits the contraction process It is well known that adrenaline in some species although usually exerting an inhibitory effect will sometimes have a biphasic or even a purely stimulating effect (without DCI) With the guinea pig uterus HERMANSEN (1961) recently observed that the action of adrenaline changed during the experiment from inhibition to stimulation Such an effect could be seen on uteri from immature animals If uteri from guinea pigs pre treated with oestrogen were used contractions could be demonstrated right from the beginning of the experiments In our experiments young rats pre treated with stilboestrol were used throughout

The histamine exp… treated isolated rat u… facts that the effect c… it did not disappear …ualely but endured for a few cycles and that it was not abolished by dibenzyline but by mepyramine showed that the histamine stimulation was qualitatively dissimilar to the adrenaline stimulation

The specificity of both adrenaline and histamine stimulation was borne

contraction height was observed, in others the inhibition tended to decrease An increased blockade could never be demonstrated

The second procedure used corresponded to the one described in the work reported above, except that the DCI-solution was usually replaced by a DCI adrenaline solution with no washing in between and that the adrenaline concentrations used were considerably lower (0 5–1 0 ng/ml against 50–150 ng/ml) With this procedure a somewhat increased inhibition was regularly obtained It was difficult, however, to judge whether the inhibition corresponded to the original adrenaline inhibition or was less – especially as the sensitivity of the uterus might well have changed during the experiment

By the procedure adopted for the experiments reported here, the larger adrenaline concentrations used after DCI would render a comparison of inhibition before and after DCI impossible, an irrelevant difficulty however, by the fact that a reversed effect was obtained

It should be emphasized that the washing period usually employed without DCI before the adrenaline or histamine additions was not an absolute prerequisite for obtaining reversal In some experiments stimulation was observed when DCI was kept as antagonist, even in the cycle preceding stimulation

B *Specific Blockade of the Reversal Effect after DCI*

HOLZBAUER & VOGT (1955) stated that if N benzyl N(β phenoxyiso propyl)-β chlorethylamine (dibenzyline R) at concentrations from 0 25 μg/ml onwards was applied to rat uterus even briefly the uterine responses to carbachol were suppressed We found that a concentration of 0 025 μg/ml applied for 8 minutes blocked the serotonin initiated contractions, although concentrations up to at least 5 μg/ml did not influence the effect of human urinary kinin

Experiments were also carried out to discover to what extent mepyramine inhibited the uterus We found that a concentration of 10 ng/ml never inhibited the muscle, serotonin or human urinary kinin being used as agonist, but 100 ng/ml give a slight reduction in contraction height

4. Discussion.

The results show that adrenaline which usually strongly inhibits the isolated rat uterus, will stimulate the DCI treated muscle in a significant number of experiments The concentrations necessary were about 100 times larger than those normally us[illegible] ... induced contractions without DCI, as mentic[illegible] were never observed The fact that [illegible]

Acta pharmacol et toxicol 1961, 18, 307–312

From The Biological Department the Pharmaceutical Institute,
University of Oslo

The Effect of Histamine on Adrenaline Inhibition of the Rat Uterus

By

Kjell Briseid Jensen and Anne Marie Vennerod

(Received June 23, 1961)

The inhibitory effects of histamine and adrenaline on the rat uterus have been previously examined quantitatively (BRISEID JENSEN & SUND 1960 a, b) Both amines inhibited various agonists to approximately the same extent, the necessary concentrations of histamine being roughly 10 000 times higher, on a weight basis, than those of adrenaline As some of the agonists used must be presumed to act on different primary structures, the results gave support to the view that neither histamine nor adrenaline acts on primary structures initiating the contraction process, but either on subsequent general stages in the process or by stimulating mechanisms that act in opposition to the structures releasing contraction

As previously emphasised (BRISEID JENSEN & SUND 1960 b), the inhibition of rat uterus by adrenaline and by histamine showed several points of resemblance, it seemed appropriate to discover, if possible, whether the two drugs affect the same structures when inhibiting the muscle The fact that dichlorophenyl isopropylaminoethanol (DCI) causes a reversal with both adrenaline and histamine is of no value as evidence for a common mechanism for a histamine reversal in prolonged experiments can also be obtained without DCI (BRISEID JENSEN & VENNEROD 1961)

In the work reported in this paper the actions of histamine and of other rat uterus inhibitory substances on the adrenaline effect were investigated A decreased adrenaline inhibition would strongly suggest that the other d...

...presses rat uterus

out by also giving papaverine as antagonist In concentrations of about 1 µg/ml this drug always inhibited the muscle

A histamine reversal without DCI necessitated a comparatively long experimental period, in our experiments at least 5 hours The fact that a single dose of mepyramine with a lasting effect again reversed the histamine stimulation suggests that the stimulating effect was similar to that obtained after DCI

Even if the results afford strong evidence that the mechanisms of stimulation by adrenaline and by histamine are different, this does not exclude the possibility that their mode of action in inhibiting the muscle may be the same

Summary.

In several experiments (9 out of 30) the inhibitory effect of adrenaline on the isolated rat uterus could be reversed by DCI (1 dichlorophenyl 2 isopropylaminoethanol) The histamine inhibition was also found to be reversed (in 13 out of 28 experiments) but the reversal by the two drugs did not always occur in parallel Whereas adrenaline stimulation was never observed in uteri not pre treated with DCI, a histamine reversal could also be obtained without the use of this drug Thus in long lasting experiments a gradual change from histamine inhibition to histamine stimulation could be observed

The adrenaline induced contractions occurred immediately after addition of the drug but the histamine acted much later Sometimes histamine stimulated only indirectly by increasing the contraction heights caused by the following agonist doses Serotonin or human urinary kinin was used as agonist

The adrenaline stimulation was blocked by dibenzyline but not by mepyramine, the opposite could be demonstrated for histamine These results show that the histamine stimulation was qualitatively different from the adrenaline stimulation Papaverine, which was used as control antagonist, always inhibited

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In the work reported in this paper the actions of histamine and of other rat uterus inhibitory substances on the adrenaline effect were investigated A decreased adrenaline inhibition would strongly suggest that the other drug acted on the same structures as did adrenaline, or on a subsequent stage in the process affected by adrenaline A decrease in inhibition would also give support to the generally accepted view that adrenaline actively depresses rat uterus

1. Technique.

The technique was as previously described (BRISEID JENSEN & VENNERØD 1961)

2. Experimental and Results.

Serotonin (5-Hydroxytryptamine) or human urinary kinin was used as agonist. As soon as the muscle preparation had become stable on a submaximal dose, adrenaline was given as antagonist at a concentration (1–4 ng/ml) sufficient to produce fairly strong inhibition, – a 70–85% reduction in contraction height. When the inhibition level was stable, the adrenaline antagonist solution was replaced by a solution containing in addition about 1 µg histamine per ml. In some instances only insignificant changes in contraction height were observed, but usually the inhibition decreased clearly. As is evident from the experiment illustrated by fig. 1, inhibition increased again when adrenaline alone was given as antagonist.

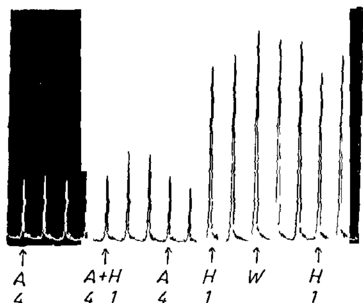


Fig. 1. The effect of histamine on adrenaline inhibition of rat uterus.
Agonist: Serotonin 6 ng/ml. Adrenaline (A) ng/ml. Histamine (H) µg/ml. Wash fluid (W).
The letters refer to the marked and the subsequent cycles.

The last contractions shown in fig. 1 demonstrate that a time conditioned histamine reversal (BRISEID JENSEN & VENNERØD 1961) was not obtained in this particular experiment: the drug still inhibited to some extent.

The effects of papaverine, mepyramine and tolazoline on adrenaline

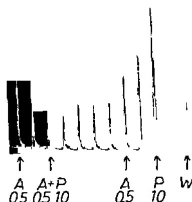


Fig 2 The effect of papaverine on the adrenaline inhibition of rat uterus
Agonist Serotonin, 12 ng/ml Adrenaline (A) ng/ml Papaverine (P) µg/ml Wash fluid (W)
The letters refer to the marked and the subsequent cycles

inhibition were examined in the same way as the histamine effect Neither papaverine nor mepyramine reduced the adrenaline effect, in fact the inhibition increased appreciably (figs 2 and 3) Tolazoline, however, reduced the adrenaline inhibition (fig 4)

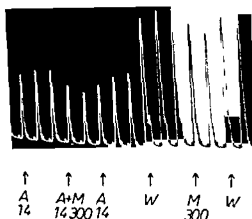


Fig 3 The effect of mepyramine on the adrenaline inhibition of rat uterus
Agonist Serotonin 4 ng ml Adrenaline (A) ng ml Mepyramine (M) ng/ml Wash fluid (W)
The letters refer to the marked and the subsequent cycles

Discussion.

In his modification of the drug-receptor theory STEPHENSON (1956) applied the term "efficacy" to agonists; *it is related to* ARIËNS & GROOT's (1954) concept of "intrinsic activity". Besides the affinity of a drug for a set of receptors, its inherent "efficacy" would thus also determine its effect. STEPHENSON strongly distinguished between agonists and antagonists, the latter possessing an "efficacy" of 0. According to such a view, when adrenaline counteracts the stimulating effects of drugs on smooth muscles, it is no true antagonist if it operates by stimulating inhibiting structures. *Substances that actively depress smooth muscles will* antagonize the effects of various stimulating substances unspecifically. GADDUM (1957) classified them as "independent antagonists", possessing "efficacy", they are, however, often considered as a kind of agonists. Though adrenaline is effective in extremely small concentrations, there may well exist numerous substances with the same mode of action, but with a low "efficacy". It may then be difficult to ascertain whether the substance in question reduces the effect of a stimulating drug as a *true* antagonist or as an agonist with the opposite effect.

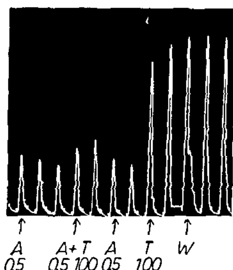


Fig. 4. The effect of tolazoline on the adrenaline inhibition of rat uterus. Agonist: Serotonin 4 ng/ml. Adrenaline (A) ng/ml. Tolazoline (T) μg/ml. Wash fluid (W). The letters refer to the marked and the subsequent cycles.

If we recognise that inhibiting substances may have inherent "efficacies", it would be permissible to classify them as *antagonists* if they have affinities only, as *active antagonists* if in addition they have "efficacies".

The results *given in this paper afford strong* evidence that, when histamine inhibits the rat uterus, it affects the same structures as does

adrenaline or has an effect at subsequent stage in the same process In concentrations that inhibited to some extent the uterus preparation, histamine evidently reduced adrenaline inhibition Experiments with papaverine and mepyramine demonstrated that those drugs presumably act on other structures, since they increased the adrenaline effect It is possible that histamine acts as a true antagonist, but there is also the possibility that histamine is an active antagonist with an exceptionally low "efficacy"

To prove this one would have to use another antagonist, acting on the same structures and able to reduce the histamine inhibition of rat uterus Such a substance would be difficult to find, it would need to have an extremely low "efficacy"/affinity ratio

NISHIYAMA & CHUMA (1955) found that tolazoline (priscol ®) did not affect the inhibiting effect of adrenaline on rat uterus, but diminished the histamine action In our experiments tolazoline was found to reduce adrenaline inhibition of rat uterus, possibly also histamine inhibition The effect on adrenaline was evident (fig 4) but the effect on histamine was less pronounced Since tolazoline at the necessary concentrations (100 μg ml) itself sometimes caused weak stimulation, sometimes weak inhibition, no clear conclusion can be drawn about whether histamine is to be classified as an active antagonist to rat uterus or not In an experiment, in which tolazoline (100 μg/ml) slightly stimulated rat uterus, it was demonstrated that this effect could not be eliminated by atropine (1 μg/ml), mepyramine (0 1 μg/ml) or dibenzyline (0 25 μg/ml for 8 minutes)

Summary.

The inhibiting effect of adrenaline on rat uterus was found to be reduced when histamine was given simultaneously at concentrations 250–1000 times higher (by weight) Since histamine itself inhibited the muscle in the same experiments the results strongly suggest that it acts on the same structures as does adrenaline or on a subsequent stage in the adrenaline-initiated process The decreased adrenaline inhibition also gave support to the generally accepted view that adrenaline actively depresses rat uterus

Experiments with papaverine and with mepyramine demonstrated that those drugs increased adrenaline inhibition, thereby suggesting actions on other structures in the muscle

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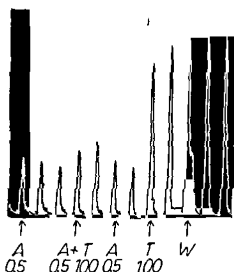


Fig 4 The effect of tolazoline on the adrenaline inhibition of rat uterus *Agonist Serotonin* 4 ng/ml Adrenaline (A) ng/ml Tolazoline (T) µg/ml Wash fluid (W) The letters refer to the marked and the subsequent cycles

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results were, however, never very pronounced, and, since tolazoline itself sometimes exerts a weak depressant effect on rat uterus, and sometimes a weak stimulation, no definite conclusions could be drawn as to the "efficacy" of histamine

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Acta pharmacol et toxicol 1961, 18, 313–320

From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller, M D)

Effect of Histamine Depletion on Formation of Granulation Tissue and Healing of Linear Wounds.

By

Eigill Hvidberg, Ole Jørgensen, Arne Schmidt and Jens Schou

(Received October 28 1961)

Recent investigations into healing of wounds suggest that histamine plays a part in this process Relatively little has been published on this subject, and the results reported seem to be contradictory

The object of our work has been to study the possible influence of histamine on healing and formation of granulation tissue, as well as to record the alterations in histamine content of wound tissue provoked by injury

Methods.

Female rats weighing about 100 g were used for all experiments, except those set out in table 3, for which the rats weighed about 160 g

Tensile strength of wound The animals were anaesthetised with ether, and the coat was shaved on the back with an electric clipper A 6 cm longitudinal incision was made along the middle of the back [illegible]
to the muscular fascia [illegible]
silk 000, Anacap ®) [illegible]
no dressing was used [illegible]
The animals were kille[illegible], and the coat was shaved again The tensile strength of the wounds *in situ* was then measured by the technique described below

Technique A strong silk suture passing through the skin only was inserted on a level with the middle of the wound at a distance of 1 cm [illegible]

Histamine depletion All the test animals were kept under standard conditions with free access to water and a commercial and adequate diet For histamine depletion we used compound 48/80, which was injected intraperitoneally once daily for 10 days, beginning with a dose of 25 micrograms and ending, after a gradual rise, with 500 micrograms daily on the tenth day On the eleventh day the animals were operated on As a maintenance dose during the experimental period, 500 micrograms were given daily In a single series (see table 3) a group of animals was depleted of histamine by means of polymyxin B, according to the schedule first day 2 5 mg/kg intraperitoneally, rising to 7 5 mg/kg On the fourth day the animals were submitted to experiment

Histamine determination The hair was cut with an electric hair clipper Next 0 6–0 7 g skin or wound tissue was excised This was immediately cut into small pieces and placed in 10 0 ml of 10% trichloracetic acid The mixture was left for 24 hours at about 7°C (shaken at intervals) The whole was then filtered through gauze, as much fluid as possible having been pressed out of the tissue by hand The filtrate now constituted 10 ml The filtrate was extracted with anhydrous ether, 3 × 40 ml for about 30 minutes each time The ether layer was removed by suction Then the aqueous layer was heated to about 60° for 1 hour, to remove any ether The total amount of fluid was now about 8 ml at pH about 5 The histamine concentration was determined biologically on guinea pig ileum For each test we used one standard and one test solution, at two doses each Each specimen was submitted to six × four determinations, from which its histamine concentration was calculated (BURN 1950) and expressed as micrograms of free histamine base per gram wet tissue The biological assays were carried out in Tyrode's solution prepared from redistilled water containing atropine, and continuously aerated by carboxygen, Ph D (96% v/v O_2 + 4% v/v CO_2) at a temperature of 37° The specificity of the histamine determination was proved by abolishing the contractions with mepyramine

Determination of water content The skin, both intact skin and the portion including the wound, was cut and depilated with a paste containing barium sulphide After washing and drying the tissue, specimens were excised and at once weighed on a torsion balance The tissue was then frozen at about −25° and dried in a desiccator over silica gel at a pressure of 1–3 mm Hg for 24 hours This procedure was repeated until the tissue reached constant weight The tissue was then defatted by shaking several times with a solvent in small perforated metal cases First light petroleum was used twice for 45 minutes each time, and then anhydrous ether for 45 minutes at a time until constant weight was reached (usually four times) The water content was expressed as gram water per 100 g dried defatted tissue

Hexosamine determination The hexosamine content was determined in dried defatted tissue, by BLIX's (1948) modification of ELSON & MORGAN's method The tissue was hydrolysed for 15 hours with 2 N-HCl in sealed glass ampoules at 100° The hexosamine content was expressed as milligram hexosamine per 100 g dried defatted tissue

Formation of granulation tissue The technique described by RUDAS (1960) was employed for this test With the rat under ether anaesthesia a circular piece of skin was excised medially and distally on the back A plastic ring was inserted in th resultant hole The diameter of the ring was so much larger than the hole that the ring was kept fixed there Further, the ring was provided with flanges to prevent its slipping off All the rings had exactly the same inner diameter (20 mm), so that quantitative analyses of the granulation tissue formed in the rings were possible The animals were killed 8 days later, and the rings were removed The granulation tissue

formed was then easily removed by blunt dissection from the underlying muscular fascia The tissue was weighed on a torsion balance immediately after excision and then freeze-dried and defatted in the manner described for skin tissue

Collagen determination In the granulation tissue the content of collagen was determined as hydroxyproline multiplied by the factor 7 46 The dried defatted tissue was hydrolysed with 6 N HCl for 24 hours at 100° in sealed glass ampoules The hydrolysate was filtered and the amount of hydroxyproline measured by MARTIN & AXELROD s (1955) modification of NEUMANN & LOGAN s method

Statistics For statistical purposes we used the t test

Results.

The difference in tensile strength between the wounds in the controls and in the histamine depleted animals during the first 10 days is seen from fig 1 After 7 days the strength seemed to be greater in the histamine-depleted animals than in the controls The difference was slight but significant ($P < 0.02$) After 10 days there appeared to be still a slight difference, but it was no longer statistically significant

To demonstrate that the treatment with 48/80 had the desired histamine depleting effect, a number of skin specimens from untreated and treated animals were examined for their contents of histamine It is seen in table 1 that histamine depletion reduced the *histamine concentra-*

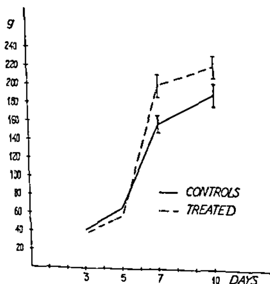


Fig 1 Tensile strength of linear wounds
Ordinate tensile strength in gram
Abscissa number of days after formation of wound The vertical lines indicate the standard errors of the mean Controls untreated treated group given compound 48/80 before the wound formation

Table 1

for water content (for further explanation see text) n = number of animals

Sample	Controls			Histamine depleted	
	n	µg/g wet weight	µg/g corrected	n	µg/g wet weight
Normal skin	19	38 1	-	30	6 0
Wound tissue first day	7	15 0	20 0	5	2 7
Wound tissue third day	7	19 9	22 9	5	2 2
Wound tissue fifth day	9	20 4	20 8	5	2 7
Wound tissue seventh day	8	21 5	21 9	5	3 2
Wound tissue, tenth day	8	28 7	28 7	-	-

tion in the skin on the average to 16% of the normal (from about 38 to about 6 micrograms per gram wet tissue) Table 1 further shows the alterations in histamine concentration of wound tissue during the post operative phase In wounds of untreated animals it was found to be only about half the normal within the first week, but it displayed a markedly rising tendency on the 10th day The water content being larger in wound tissue, a correction had to be made to render the results comparable The second column of histamine values in table 1 shows the corrected histamine concentration at constant water content The figures show that during the first post operative week the histamine content was fairly constant at just over half the normal for skin tissue in this region In histamine-depleted animals the already low histamine content in the wounds was halved

The *contents of water and hexosamine* increased considerably in wound tissue already within the first 24 hours (DUNPHY & UDUPA 1955, SCHMIDT 1961) Table 2 illustrates this fact for both normal and histamine depleted animals There seemed to be some difference between the two groups The first day the contents of both water and hexosamine were small in the wound tissue of the histamine depleted animals ($P < 0\ 02$), compared with the values in normal animals, whereas this was not so on the fifth post operative day This could hardly have been due to a systematic change in the water and hexosamine contents of the connective tissue during the histamine depletion, since table 3 shows, for another group of test animals, that no unequivocal change takes place in normal skin tissue during such treatment

Table 4 records a marked reduction of both the dry and the wet weight of granulation tissue after histamine depletion ($P < 0\ 001$)

Table 2.

Contents of hexosamine and water in linear wounds of histamine-depleted and control rats. The contents of water and hexosamine are given in terms of defatted dry weight n = number of animals

	Controls			Histamine depleted		
	n	Water g/100g	Hexosamine mg/100 g	Water g/100 g	Hexosamine mg/100 g	n
Normal skin	31	263	424	259	424	6
Wound tissue ½ day	10	359	608	–	–	
Wound tissue, first day**	17	352	631	331	588	13
Wound tissue, third day	14	305	560	–	–	
Wound tissue, fifth day	12	267	542	280	553	16
Wound tissue, seventh day	20	268	521	–	–	

** Significant difference between treated and control animals

Table 3.

Contents of water and hexosamine in intact rat skin (rats weighing 160 g) after treatment with 48/80 or polymyxin No significant difference was found between the treated and the untreated n = number of animals

	n	water g/100 g dry defatted tissue	hexosamine mg/100 g dry defatted tissue
Controls	20	217	404
Treatm with 48/80	18	217	392
Treatm with polymyxin B	8	217	382

Table 4.

Changes in granulation tissue (according to Rudas) after histamine depletion The wet and the dry defatted weight are given in milligrams, contents as g/100 g dry, defatted tissue, hexosamine expressed as mg/100 g dry, defatted tissue s e m = standard error of the mean

	Granulation tissue								
	n	wet weight mg	s e m	dry defatted weight mg	s e m	collagen g/100 g g/100 g	s e m	n	hexosamine
Controls	17	450	22	72	±3	13 72	±0 64	10	1084
Histamine depleted	17	332	20	51	±3	14 38	±0 38	7	1075

The figures for the collagen content, on the other hand, seemed a little higher after histamine depletion, but the difference was not significant. The amount of hexosamine in the granulation tissue was found to be totally unaffected by histamine depletion.

Discussion.

Studies of histamine metabolism in embryonic tissue have shown a relationship between growth activity and histamine production (KAHLSON 1960) These studies led to investigations into the significance of histamine in the healing of wounds (KAHLSON *et al* 1960) The production of histamine was found to be increased in cutaneous wounds, but the concentration of histamine in the wounds (only measured on the fifth day, was reduced Treatment with the histamine liberator, compound 48/80 (a condensation product of para-methoxyphenyl ethyl-methylamine with formaldehyde), reduced the histamine content of the wounds, but enhanced histamine production At the same time the tensile strength of the wounds increased This suggests that the production of histamine is a factor influencing the rate of cutaneous wound healing The results of BOYD & SMITH's (1959) investigations contrast with this They found that administering compound 48/80 inhibited the healing process, whether estimated by histological methods or by measurements of the tensile strength The experimental conditions in the two investigations, however, were not identical, KAHLSON *et al* treated the animals for only 3 days pre-operatively, whereas BOYD & SMITH pre-treated them for a period of 10 days FIORI DONATI & MOLTKE (1960) found no change in tensile strength of wounds in response to treatment with Polymyxin B, a specific histamine liberator, despite degranulation of the mast cells indicating liberation of histamine Reserpine, on the other hand, was found to reduce the strength of the wound, possibly owing to liberation of serotonine

Both BOYD & SMITH and KAHLSON *et al* treated the animals with histamine compounds that slowly gave off free histamine According to KAHLSON *et al*, subcutaneous injection of a histamine dipicrate suspension in oil had no effect on healing, as measured by tensile strength BOYD & SMITH, on the other hand, after intraperitoneal injection of histamine phosphate, observed histological signs of increased healing and a tendency towards stronger wounds

In agreement with KAHLSON *et al*, we found increased strength of wound after histamine depletion, though not until the seventh day, whereas they observed increased strength from the fifth day However, no appreciable permanent increase in strength seemed to be obtained in the histamine-depleted animal, the stimulating effect on healing having already worn off on the tenth day

In the first phase of healing, the exudative phase, the reactive oedema seemed to be less pronounced (table 2) in histamine depleted animals than in controls, the amounts of hexosamine and water in the wounds being smaller during the first few days The rise in hexosamine concen-

tration is supposed to indicate exudation of protein containing fluid from the blood stream (JACKSON, FLICKINGER & DUNPHY 1960, SCHMIDT 1961) We cannot at present say for certain whether reduced exudation plays any part in the strengthening of the wounds during histamine-depletion However, if liberation of histamine is regarded as a necessary link in the development of traumatic oedema (cf WEGELIUS & ASBOE HANSEN 1956) decreased post-operative exudation in the histamine depleted animals is consistent with this hypothesis In this connection the wound must be regarded as an injury KAHLSON *et al* and BOYD & SMITH agree with us that histamine concentration falls appreciably in wounds, even when values are corrected for alterations in water content (see table 1) The liberation of histamine in wounds is indeed so large that the low histamine content of the skin after histamine depletion is halved again in wound tissue

In histamine depleted animals the tensile strength of linear wounds was found to be increased The weight of granulation tissue (wet as well as dry) on the other hand was seen to be significantly reduced These facts seem on a first view to be contradictory However, on examining the collagen content of granulation tissue, we found it to appear somewhat larger in histamine depleted animals than in controls, though the difference was not statistically significant The hexosamine concentrations were identical in the two groups

It is hardly possible, on the basis of our present knowledge, to explain the difference between the effects of histamine depletion on linear wounds and on granulation tissue The effect of histamine on the process of healing is still in many ways obscure The fact that the results are contradictory in several respects perhaps supports the view that histamine is of no decisive importance in the healing of wounds, but may play a secondary part We are still in doubt as to what biochemical factors are essential to the processes of healing

Summary.

The effects of histamine depletion on linear wounds and granulation tissue have been studied The tensile strength of linear wounds was found to be increased in histamine depleted rats, whereas the formation of granulation tissue was inhibited as estimated by the weights of dry and wet granulation tissue The amounts of hexosamine and collagen in granulation tissue were seen to be uninfluenced by histamine depletion

The histamine concentration in wound tissue was reduced, compared with that in intact skin In the histamine depleted animals, this low histamine concentration was halved

Acknowledgements

Part of this investigation was carried out in The Laboratory for Investigation of the Pharmacology of Connective Tissue with a grant from the Danish National Association against Rheumatic Diseases

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Acta pharmacol et toxicol 1961, 18, 321–328

From the Research Institute of National Defence, Dept 1, Sundbyberg 4
and the State Veterinary Medical Institute, Stockholm 50, Sweden

The chronic Toxicity of N-Methylpyridinium-2-aldoxime Methane Sulphonate (P2S), a Reactivator of phosphorylated Cholinesterase.

By

Lennart Albanus, Bertil Järplid and Anders Sundwall

(Received August 29, 1961)

Certain oximes are effective as antidotes in organophosphorus anticholinesterase poisoning N methylpyridinium-2 aldoxime (= pralidoxime WHO, NFN) has been shown to be effective in experimental and accidental poisoning in man (NAMBA & HIRAKI 1958, KARLOG *et al* 1958 and GROB & JOHNS 1958) and is claimed to be safely tolerated in single doses (JAGER & STAGG 1958) Further, evidence has been obtained that the oxime may be used prophylactically (EDERY & SCHATZBERG-PORATH 1959, COLEMAN *et al* 1961) It could thus be of importance to have information about the chronic toxicity of the drug

Materials and Methods

[illegible]

[illegible] groups were injected with P2S for five days in a week and one control group with saline The drug unsterilized was dissolved in saline and injected intramuscularly in a volume of 2 ml per kg body weight for 11 weeks The animals were weighed every week

Rabbits of either sex weighing 1 7–2 5 kg were divided into 3 groups of 5 animals Two groups received P2S intramuscularly for five days in a week, and one group received saline (the injected volume being 1 ml per kg body weight) The animals were weighed weekly throughout the experiment, which lasted 9 weeks Blood samples were collected by heart [illegible]
and leucocyte cour[illegible]
sodium and non p[illegible]
trophoresis (according to VALMET & SVENSSON (1954) and ARONSSON & GRÖNVALL (1957))

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[illegible] & SVENSSON (1954) and ARONSSON & GRÖNVALL (1957))

Dogs In these experiments P2S was administered orally in gelatin capsules for five days in a week for 17 weeks Three dogs weighing 13–17 kg were used, and the daily dose was one g Blood samples were taken weekly, and serum was analysed for glutamic oxalic acid transaminase (GOT) and glutamic pyruvic acid transaminase (GPT) activities (method of ORDELL) Determinations of haemoglobin, erythrocyte and leucocyte counts, toxic granulation of erythrocytes, serum proteins, non protein nitrogen, potassium, sodium and bicarbonate were made

The rabbits were fed on hay, oats, carrots and water ad libitum Rats and dogs were fed on commercial diets SHP and Doggy, respectively (HOLMBERG & NELSON 1957, BJÖRCK 1961)

Dogs and rabbits were killed by intravenous injection of sodium pentobarbital and rats by inhalation of chloroform Post-mortems were made immediately after death Material for histological examination was taken from liver, kidney, heart, skeletal muscle, spleen, lymph nodes and bone marrow from all animals, and from lung, brain, adrenal and thyroid gland also from some In rats and rabbits the site of injection was examined, and in the orally treated dogs all parts of the gastrointestinal tract were examined histologically The material was fixed in 10% formaldehyde, embedded and stained with haematoxylin and eosin, periodic acid Schiff reaction (PAS) with and without previous treatment with diastase, van Gieson's stain and Hueck's modification of Tirmann Schmelzers turnbull blue method Frozen sections were stained with scarlet red and haemalum

Results

Rats P2S given in doses of 50 and 150 mg per kg body weight for 11 weeks did not retard the growth of young rats (fig 1) These doses correspond to a total accumulated dose of about 11 and 33 LD50, respectively One rat in the group receiving the highest dose died after two weeks

All rats were subjected to post-mortem On gross examination all appeared in a normal state of nutrition, no pathological changes being seen No histopathological changes could be detected in heart, liver, kidney, spleen, lymph nodes or bone marrow No bacteria could be cultured from liver or spleen of the rat that died spontaneously

Rabbits were injected intramuscularly with P2S 50 and 100 mg per kg for 10 weeks corresponding to a total accumulated dose of 10 and

2

f

summarised in table I As seen, except for the leucocyte counts, no differences between the control group and the P2S treated groups could be detected

At post-mortem most of the rabbits were in a state of nutrition somewhat beneath normal, probably as a result of infestation with coccidia in liver and intestine Pathological changes at the site of injection were seen in nine out of the ten rabbits treated with I

Table I

ell counts and chemical analyses of blood from rabbits receiving pralidoxime methane ulfonate (N methylpyridinium 2 aldoxime methane sulphonate — P2S) intramuscularly for ten weeks (Figures represent mean and extreme values)

Group	Haemoglobin (gram per 100 ml blood)	Erythrocytes (millions per mm³)	Leucocytes (thousands per mm³)	Total protein	Albumin
				(gram per 100 ml serum)	
Control	10 7	5 5	7 6	5 3	3 8
	(9 8–12 1)	(4 7–7 2)	(5 0–13 0)	(4 1–5 8)	(3 1–4 2)
P2S 50 mg/kg	11 2	5 1	7 8	5 6	4 0
	(10 5–12 1)	(3 9 5 9)	(5 1–12 0)	(5 2–6 2)	(3 4–4 4)
P2S 100 mg/kg	11 0	5 2	5 7	5 7	4 1
	(10 6–11 6)	(4 5 5 6)	(2.9–7 5)	(5 2–6 4)	(3 4–4 5)

Group	Globulin	Albumin/ Globulin	Non protein nitrogen (mg per 100 ml blood)	Potassium (m eq/l serum)	Sodium (m eq/l serum)
	(gram per 100 ml serum)				
Control	1 50	2 65	34	4 9	137
	(1 0–2 1)	(2 18 3 23)	(30–36)	(4 0–6 2)	(132–141)
P2S 50 mg/kg	1 68	2 41	38	4 5	134
	(1 3 1 9)	(1 79–3 23)	(30–42)	(3 7–5 0)	(131–137)
P2S 100 mg/kg	1 66	2.55	34	4 3	135
	(1 3 2 1)	(1 80–3 30)	(32–38)	(3 9–4 7)	(131–137)

controls Histological examination showed varying degrees of haemorrhage and purulent and indurative myositis No histopathological changes were found in heart, spleen, lymph nodes or bone marrow Casts with erythrocytes were found in the collecting tubules of the kidney in three controls and in nine of the treated animals, but no degenerative changes could be detected in the tubular epithelium Similar changes were observed in another group of 15 apparently normal rabbits In the livers of one rabbit from each of the two treated groups the central veins and the central parts of the sinusoids were dilatated and the hepatic cell cords were narrow, with reduced staining In these hepatic cells there was loss of cytoplasmic basophilia and reduced content of material with positive PAS reaction, which could be eliminated by diastase digestion The nuclei here showed some pycnotic changes In the liver of the rabbit that had received the highest dose, fat droplets were seen both in parenchymal cells and Kupffer cells

Dogs Preliminary experiments showed that about 500 mg P2S per kg body-weight administered orally is a lethal dose The plasma concentrations measured shortly before death were in the range of 60 to 90 µg per ml

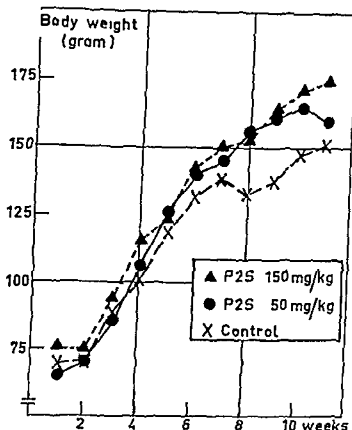


Fig 1 Average weekly body weights of rats receiving P2S intramuscularly

For the chronic toxicity study 60 to 70 mg P2S per kg were administered orally in gelatin capsules for 17 weeks this corresponds to a total accumulated dose of about 10 LD50 The plasma concentration measured two hours after administration, to be sure of absorption, was 19 to 20 µg/ml *Symptoms from the gastrointestinal tract or symptoms due to systemic effects were not seen*

Haemoglobin and cell count were determined on three occasions during the experiment and non-protein nitrogen, bicarbonate, sodium, and potassium shortly before killing All results obtained were within the *normal range Serum glutamic oxalic acid transaminase* (GOT) and glutamic pyruvic acid transaminase (GPT) activity were measured weekly, all values were within the range of normal variation (CORNELIUS *et al* 1959) Serum proteins were determined a few days before slaughter The results are summarized in table 2 In all dogs the albumin values obtained were below those found in ten of our untreated dogs In one dog the γ globulin fraction was increased to 3 4 g per 100 ml of serum The urinary sediment was normal, and no protein or glucose was found

At post-mortem *one dog (n o 2) was found to be in a poor state of nutrition* There were neither gross nor microsco



Fig 2 Photomicrograph of the stomach (fundus region) from a dog treated orally with P2S for 17 weeks Note fibrosis of the propria (Htx – eosin) × 150

liver, spleen, lymph nodes or bone marrow In the intestine we found a moderate number of roundworms (*Toxascaris leonina*), and in kidneys and pancreas there were granulomatous nodules caused by larva migrans All the dogs had histopathological changes in the mucous membrane of the stomach. In one (n o 1) there was a moderate fibrosis in the cardiac region, with local atrophy of the glands In the fundus region of this dog there were superficial fibrotic scars and a moderate fibrosis in the propria of the mucous membrane (fig 2) In the pyloric region of the same dog there was a slight fibrosis throughout the mucous membrane The other

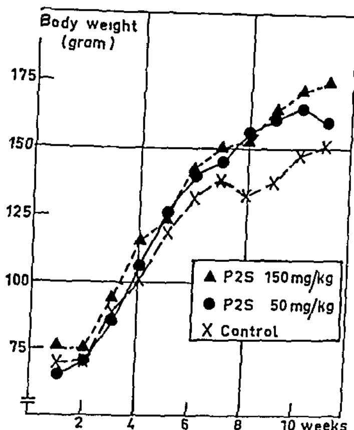


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Table 2.

Analysis of serum proteins from dogs treated with P2S 60–70 mg/kg orally for 17 weeks Figures indi[illegible] [illegible] of VALMET & SVENSSON (1[illegible] 1957) Tota[illegible]

	Total protein	Albumin	Globulin			Albumin/Globulin
			α_1–α_3		β_1–β_2	
Dog 1	6 2	2 0	0 6	0 4	3 4	0 45
Dog 2	5 6	3 1	0 6	1 2	0 6	1 29
Dog 3	4 8	2 2	0 9	1 1	0 6	0 85
Untreated dogs *Mean and* standard *error n = 10*	7 3±0 2	4 4±0 1	1 05±0 07	1 4±0 1	0 6±0 05	1 5±0 1

two dogs had slight *superficial fibrosis in the cardiac and pyloric regions* respectively. No correlation between the degree of fibrosis and the state of nutrition was found.

Discussion.

The experiments show that fairly large doses of pralidoxime methane *sulphonate (P2S) can be administered for long periods to dogs,* rabbits and rats without producing serious side effects By studying growing rats during 50 days treatment (total cumulative dose, 1 5 LD50) and by subsequent histological examination, COLEMAN *et al* (1961) found no signs of toxic lesions However, in our study of higher doses and with a more detailed examination of the treated animals, we have found toxic manifestations that cannot be ignored when considering the prophylactic *use of the oxime*

The changes in the gastric mucosa of the orally treated dogs must be a local effect of the drug There was no cellular infiltration in them, and they were not similar to *those described as pre-cancerous lesions in man* (WILLIS 1953) Possibly the changes has begun as oedema, which we have seen in a dog lethally intoxicated with P2S orally Local changes were also found in the rabbits treated intramuscularly

The significance of the decrease in leucocyte count found in the rabbits receiving the highest dose of P2S is difficult to evaluate, since the differential cell count was not changed and a wide normal variation has been reported (SCHERMER 1958) *In the dogs no such changes were detected*

The low serum albumin values found in the dogs may be attributed to interference with protein metabolism Impairment of kidney function is not probable, as no protein was *found in the urine* ARMSTRONG *et al*

(1960) have shown that serum albumin labelled with ^{131}I passes from the circulation of rabbits into the duodenum Possibly this leakage may be increased during oral P2S treatment, as occurs in the so called protein-losing gastroenteropathy (JARNUM & SCHWARTZ 1960) The fact that normal serum protein values were found in the rabbits supports this interpretation Protein synthesis in the liver may also be affected, especially as an abnormal increase in the γ-globulin fraction was found in one dog The fact that no significant increase in the weekly observed serum GPT and GOT activity was found in the dogs excludes, however, severe tissue damage (CORNELIUS *et al* 1959, BALAZS *et al* 1961)

The unspecific histopathological changes detected in the liver of two P2S treated rabbits were not pronounced, and it is uncertain whether or not they were due to the administered drug

In most of the rabbits casts with erythrocytes were found in the collecting tubules of the kidneys These changes were about equally distributed between controls and P2S treated animals, we have seen similar changes in another group of fifteen untreated rabbits from our own stock This seems to us a remarkable pathological finding

The results obtained in our investigation show that P2S given in large doses for a long time causes local changes at the site of administration and that interference by the drug with protein metabolism cannot be completely excluded

Summary.

The chronic toxicity of pralidoxime methane sulphonate (N methyl-pyridinium 2 aldoxime methane sulphonate = P2S) has been studied in rats, rabbits and dogs

P2S was administered intramuscularly to rats and rabbits and orally to dogs for 9 to 17 weeks The total accumulated doses were 10 to 30 LD50

The drug caused local changes, such as fibrosis of the mucous membrane of the stomach in the dogs and myositis at the site of injection in the rabbits No major histopathological changes in other organs reasonably to be attributed to the drug were detected

Interference with protein metabolism was indicated by the fact that abnormal serum protein values were found in the dogs No other significant changes in blood morphology or blood chemistry could be detected

Acknowledgement

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(Prof Ernst Bárany, M D)

Theophylline Diuresis in the Chicken

By

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(Received September 16 1961)

BARTRAM (1932) and CHRISTIAN & BARTRAM (1932) have investigated the effects of xanthine diuretics by direct administration into one renal artery of the dog, whence the drugs, by virtue of the nature of mammalian renal blood supply, were delivered to both the glomeruli and the tubules

In the study reported here we used the SPERBER chicken preparation, aminophylline (WHO, BP) = theophyllamine (NFN) and theophylline were introduced into the renal portal circulation via one leg vein Thus the first impact of the introduced substances was mainly on the renal tubules of the kidney on the injected side These experiments have shown an ipsilateral excess of electrolyte and water output, adding to the probability that the effect of xanthines is at least in part on the tubules Finally, since theophylline can behave as a weak acid and the secretion of uric acid can be reduced by probenecid in the chicken (NECHAY & NECHAY 1959, BERGER *et al* 1960), the effect of probenecid on aminophylline diuresis was examined Nothing is known about tubular handling of methyl xanthines

Methods.

The subjects were unanaesthetized Rhode Island Red hens, about 2 years old, weighing 2 0–3 5 kg

The birds were m
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[illegible] work was supported in part by grants from the Medical Faculty of the University of Uppsala Sweden and the Svenska Sällskapet för Medicinsk Forskning

original SPERBER (1948, 1949) technique was used for urine collection Small plastic funnels were sutured around the orifice of each ureter under topical lidocaine = xylo cain ® (5% ointment) anaesthesia, just before each experiment to permit separate collection of urine from each side A constant flow of distilled water, about 1 ml/min, was directed through the ureteral funnels so as to dilute the urine immediately it emerged from the ureters Thus diluted the urine dripped directly into the tubes inserted in a fraction collector equipped with a precise timing device Since the urine volume was determined by subtracting the rinsing volume from the total volume of the sample and the electrolyte concentration from the total of diluted urine, a small leak could have a profound effect on the measurement of urinary flow, though the error in the determination of electrolyte excretion would remain negligible

All birds used were selected for the ability to excrete phenol red predominantly on the injected side, as judged by visual observation of colour in the urine For this test 0 1–0 2 mg/kg of phenol red, as a 0 1% solution, was given in the course of about 2 minutes into one leg vein A few hens that failed to excrete an appreciable excess ipsilaterally were discarded

The drugs, in saline solutions, were injected or infused by motor-driven syringes through polyethylene catheters inserted into the leg vein or wing vein, as indicated in the tables and figures Theophylline was always used at concentrations of 8 mg/ml and aminophyline at 27 mg/ml

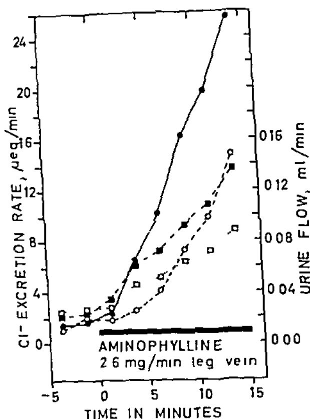
p-Aminohippurate (PAH) was infused at 0 2 mg/min as 3 mg/ml solution in saline through a similar catheter in the opposite wing vein after a priming dose of about 2 mg For equilibration 30 to 80 minutes were allowed

Methods of analysis are listed below Cl^- Potentiometric silver nitrate titration (KELLOG *et al* 1952) Na^+ and K^+, Eppendorf flame photometer Phenol red Entire urine sample diluted with equal volume of 1 0% aqueous solution of Na_2CO_3 [sodium carbonate] and shaken intermittently until all precipitate dissolved Optical density then determined in Zeiss Elko II colorimeter with Zeiss Opton filter S 53 51 PAH Method of BRATTON & MARSHALL (1939)

Results.

A *Unilateral Leg Vein Infusion of Aminophylline*

Pilot experiments showed that unilateral aminophylline infusion of 0 3, 0 7 and 1 3 mg/min for up to 30 min produced no significant diuretic effect on either side With the infusion rates of 3 9 and 5 2 mg/min aminophylline the urinary flow increased almost immediately, showing a small excess on the infused side at the former and about equal output from both kidneys at the latter dose Fig 1 illustrates the mean Cl excretion rate and urinary flow in 8 experiments, when 2 6 mg/min of aminophylline (equivalent to 2 0 mg/min of theophylline) was infused for 15 minutes into the vein of one leg, chosen at random In each 2 5 min collection period of each experiment the Cl^- excretion rates and urinary flows were higher on the infused side after the onset of diuresis The means of Cl^- excretion-rate ratios of infused side over control side for each period are shown in the right column of table 2 (At low excretion-



of

Cl control side
urine flow, infused side
urine flow control side
The means of individual Cl ratios of infused side over control side are shown in right hand column of table 2

rates close to the limits of analytical measurement ratios are unreliable) They had a tendency to become smaller toward the end of aminophylline infusion and declined still further after the infusion was discontinued, while the drug was being distributed in the body, and the diuretic effect declined more rapidly on the infused side

B *Unilateral Leg Vein Infusion of Theophylline*

To eliminate any possible artifacts due to ethylenediamine used as a solubilizing agent in aminophylline, theophylline as such was also tested

Table 1

Effects of single wing vein doses of theophylline on Cl and urine output

Exper Number	Theophylline into wing vein mg/kg	Peak excretion rate				Total output in 1 hr			
		Cl, μeq/min		Urine ml/min		Cl, meq		Urine ml	
		Right kidney	Left kidney	Right kidney	Left kidney	Right kidney	Left kidney	Right kidney	Left kidney
1*a*	10	8	7	0 07	0 07	0 39	0 34	3 9	3 8
1*b*	10	24	26	0 30	0 29	1 39	1 26	12 4	11 3
2*a*	10	25	20	0 35	0 44	1 02	0 96	11 3	15 5
2*b*	10	39	34	0 44	0 42	1 72	1 43	17 2	21 1
3	10	17	12	0 13	0 14	0 53	0 48	5 0	6 3
4	10	12	12	0 09	0 09	0 44	0 44	3 4	3 3
5*a*	20	20	23	0 23	0 32	0 45	0 47	6 1	7 4
5*b*	20	58	53	0 54	0 57	1 41	1 23	13 4	13 3

Letters *b* indicate repeat experiments on same birds with corresponding *a* numbers The control values were Cl, 1 μeq/min or less urine flow 0 01–0 06 ml/min They were somewhat larger in the *b* experiments

for unilateral diuretic effect Here theophylline was infused into one leg vein at 2 0 mg/min (equivalent to 2 6 mg/min aminophylline) for 15 minutes Urinary flow, Cl^- and PAH were measured in each period of each experiment, the mean values for 6 runs are reported in fig 2 As in the analogous aminophylline experiments, there was always an excess of Cl^- excretion and urine flow on the infused side after the onset of the effect, with the ratio of infused side rate to contralateral side rate declining toward the end and after the infusion The time of onset and the extent of diuresis were about equal for theophylline and aminophylline

Just at the onset of diuresis an approximately equal bilateral rise of about 40% in PAH excretion was followed by a sharp drop From then on the output of PAH remained somewhat lower on the infused side until 15 minutes after the end of infusion

In 2 control birds (not shown), when saline infusion was substituted in equal volume and time for that of theophylline, Cl excretion and urine flow remained unchanged No changes in PAH excretion similar to those after theophylline were seen However, the number of these experiments was too small to determine whether or not the unilateral depression of PAH excretion on the theophylline infused side after the initial bilateral peak was a simple dilution effect

In 3 other controls (not shown) a unilateral infusion of theophylline did not appear to alter the symmetry of phenol red excretion after a single dose of 1 5 mg/kg given in the pectoral muscle one hour before infusion However, in 2 of these experiments the downward trend in phenol red output was arrested for about 5 minutes at the beginning of theophylline

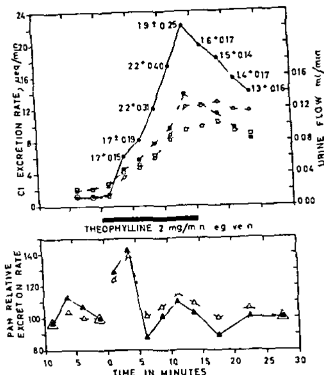


Fig 2 Effects of unilateral leg vein infusion of theophylline on urinary excretion of Cl, water and PAH Each point located at the middle of collection periods represents the mean value for 6 experiments PAH was infused into the wing vein at 0 2 mg/min throughout PAH values are given as percentage of the excretion rate in the period just before theophylline infusion The figures in the diagram indicate the means and their standard errors of individual Cl excretion rate ratios for each period

●——● Cl infused side ○——○ Cl control side

■ ■ urine flow infused side □ urine flow control side

▲——▲ PAH infused side △ △ PAH control side

infusion In the third there was a transient bilateral rise of about 40% in phenol red excretion at the beginning of infusion It appeared similar in extent to the large initial rise in PAH excretion observed in fig 2

For the same rate of Cl excretion the urinary Cl concentration was higher on the side of infusion than on the opposite side This could be seen clearly only in those experiments in which the urinary flow measurements were most reliable and the output of Cl covered wide and overlapping ranges on both sides Moreover, the response had to be large enough to permit accurate analyses at different times of infusion The two best examples of such plots are shown in fig 3 In several other experiments with results included in fig 2 a few points indicated that this was probably a regular occurrence

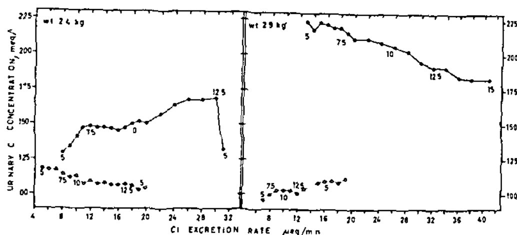


Fig 3 The relationship of Cl excretion rate to its urinary concentration during unilateral leg vein infusion of theophylline Figures in the diagram indicate time in minutes of theophylline infusion at the rate of 2 mg/min
●———● infused side ○------○ control side

C *Single Wing Vein Dose of Xanthines*

After a single dose of 10–20 mg/kg theophylline into the wing vein, the peak excretion rate of Cl^- and water always occurred in the same period on both sides The same was true for total Cl and urine output over one hour (table I) In this situation the Cl and volume excretion ratios thus approximated to unity, in sharp contrast to the clearly evident excess of ion and water diuresis when xanthines were infused unilaterally into a leg vein (fig 1,2)

With an acute wing vein injection of theophylline or aminophylline a biphasic diuretic response was observed at times, similar to that shown in fig 4 B Thus in theophylline experiments, 1a and 3 of table 1, there were 2 peaks in Cl and urine excretion separated by a dip of about 20 minutes duration Such biphasic responses occurred twice in four birds treated with aminophylline – once in two experiments at 27 mg/kg, and once in the only test performed at 67 mg/kg In the only experiment at 50 mg/kg the curves did not rise to a second peak

D *Aminophylline pre- and post-treatment with Probenecid*

a *Effect of unilateral probenecid infusion on phenol red excretion* Probenecid infused into one leg vein at 1 mg/min unilaterally depressed by about 50% the excretion of phenol red in 3 birds (fig 4A)

Other probenecid doses tested were 0 1 mg/min given once for 20 minutes with no visible effect, 0 25 mg/min for 20 min twice with a small unilateral effect, 0 5 mg/min for 20 min four times, in two of which the phenol red excretion ratio of infused to control side declined to approxi-

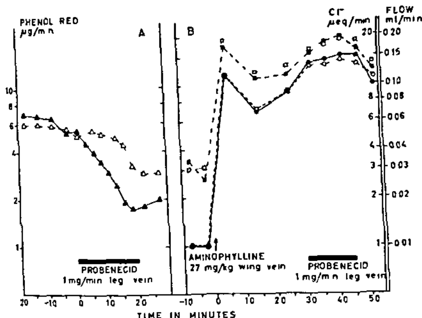


Fig 4 A Effects of unilateral leg vein infusion of probenecid on urinary phenol red excretion Phenol red was given in a single dose of 1 5 mg into the pectoral muscle one hour before probenecid infusion The points plotted correspond to the middle of collection periods ▲——▲ infused side △ --- △ control side

B Effects of unilateral leg vein infusion of probenecid on diuresis due to a single wing vein dose of aminophylline ●——● Cl infused side ○-- -○ Cl control side ■——■ urine flow infused side ⌴ ---- ⌴ urine flow control side

mately 0 7, though in the other two experiments the effect was less pronounced, 2 mg/min was tested once, an immediate and short (2–3 minutes) unilateral depression occurring before the output of phenol red was markedly and about equally reduced on both sides

b *Unilateral probenecid infusion during aminophylline diuresis* In 3 birds given aminophylline into the wing vein 30 minutes previously, the parallel excretion rates of Cl and water from both kidneys continued virtually unchanged during a unilateral leg vein infusion of probenecid at 1 mg/min for 15 minutes (fig 4 B) Two of these birds had received 27 mg/kg aminophylline and one 67 mg/kg The total Cl^- excretions during the 15 minutes of probenecid infusion were 0 22, 0 13 and 0 75 meq on the infused side, compared with 0 20, 0 14 and 0 76 meq on the control side The respective total urine volumes were 2 6, 1 7 and 4 7 ml (infused side), compared with 2 7, 1 6 and 4 5 ml (control side) Thus probenecid had no effect

c *Unilateral aminophylline infusion in probenecid pre-treated birds* Table 2 records 3 experiments in which 2 6 mg/min aminophylline were

Table 2.

Effects of unilateral leg vein aminophylline infusion on urinary Cl^- excretion in probenecid pre treated birds

Time	Cl^- excretion rate µeq /min						Mean of ratios *infused side* control side	Mean of ratios* *infused side* control side in controls of fig 1
	Weight of bird, kg							
min	2 4		3 6		2 9			
	I	C	I	C	I	C		
−60	*Probenecid 50 mg/kg wing vein as 5% solution*							
(−5 0)-(−2 5)	1	1	2	2	1	1	1 0	1 1±0 14 (8)
(−2 5)-0	1	1	2	2	2	2	1 0	1 0±0 16 (8)
	Aminophylline 2 6 mg/min leg vein infusion as 3 9% solution							
0– 2 5	2	1	18	4	3	1	3 2	1 5±0 26 (8)
2 5– 5 0	4	1	51	15	7	2	3 6	3 7±1 14 (8)
5 0– 7 5	5	2	70	29	10	5	2 3	4 5±1 59 (8)
7 5–10 0	6	2	95	47	12	8	3 2	4 2 –1 59 (8)
10 0–12 5	7	2	101	66	20	13	3 2	2 8±0 54 (8)
12 5–15 0	8	3	107	83	24	16	1 8	3 0 0 92 (8)
	Infusion stopped							
15 0–17 5	3	2	96	90	29	20	1 5	1 7±0 99 (6)
17 5–20 0		–	57	68	20	15		1 9 0 42 (4)
20 0–22 5		–	59	68	–			1 9 +0 42 (4)

I – infused kidney
C – control kidney
* *With standard errors of the means Figures in parenthesis indicate number of ratios determined*

infused into one leg vein for 15 minutes in birds that had received 50 mg/kg of probenecid intravenously into the wing one hour before Such a dose of probenecid and timing have always reduced the diuresis due to mercurials in chickens (CAMPBELL 1959) The results indicate that the ratios of Cl^- excretion between the two sides did not differ essentially from those obtained in the absence of probenecid The absolute responses in the 3 experiments differed widely, but did not indicate any inhibition of diuretic response (cf fig 1)

Discussion.

Unilateral leg vein infusion of aminophylline or theophylline resulted in an excess of diuresis on the infused side, whereas both kidneys responded about equally to wing-vein administration of these agents This was probably due to a tubular point of attack However, the unilateral nature of the response does not absolutely exclude a vascular point of attack since the arterioles imbedded in the parenchyma may also be unilaterally affected, although this seems unlikely The parallelism of all changes in PAH excretion indicated that they were caused by theophylline reaching

the systemic circulation and were thus the result of changes in blood pressure or arteriolar tone Hence, in all probability, any changes in glomerular filtration rate, which were not measured in this study, would have also occurred symmetrically with an equal effect on both kidneys

The large peaks in excretion of PAH and phenol red (which is not limited by blood flow) at the onset of unilateral theophylline infusion were probably due to a sudden bilateral displacement of urine from the dead space A simultaneous and proportionally similar rise was present in water and Cl curves when they showed a bilateral peak (fig 2)

Since large fluctuations in PAH excretion after the initial rise were not reflected in the course of diuresis, it is doubtful whether slightly reduced PAH excretion on the theophylline infused side, if it really indicated a reduced tubular blood flow, could account for the unilateral excess of diuresis The lower excretion of PAH on the infused side could possibly be due to opening of the smooth muscle sphincter (SPERBER 1949), thus shunting some blood away from the renal portal vein[3] The unilateral reduction in PAH output could not have been due to simple dilution, since unilateral saline infusion did not change the symmetry of PAH excretion Further, no specific influence of theophylline was indicated on weak acid transport, since unilateral theophylline infusion did not change the parallel course of urinary phenol red excretion

When Cl concentrations on the two sides were compared at equal Cl excretion rates rather than at the same times, urine was found to be more concentrated on the theophylline infused side than on the control side at equal excretion rates of Cl^- This seems to indicate that the diuresis was due to two effects operating to different extents in the two kidneys There could be a common bilateral vascular effect and a predominantly tubular effect on the infused side Less likely, the common effect could be due to filtered drug acting from the lumen of the nephron

Further, the biphasic response obtained during some experiments in which xanthines were given into the wing vein could indicate a mixture of two mechanisms responsible for diuresis

Since the diuretic effect of theophylline, which can behave as a weak acid appeared to be predominantly tubular in origin, and since probenecid depressed tubular secretion of uric acid (NECHAY & NECHAY 1959, BERGER *et al* 1960) the question arose whether or not probenecid would interfere with xanthine diuresis This was shown not to be so in our study, when probenecid was given before aminophylline or at the height of the diuretic response to aminophylline Hence it appeared that probenecid did not prevent the entry of aminophylline into the tubular cells and that it

[3] Theophylline at 5 10 and 20 mg/kg into the wing vein lowered carotid blood pressure in 6 experiments at each dose and thus presumably would relax a vascular sphincter

did not displace the latter from the tubular cells at all or sufficiently to affect the renal response to aminophylline[4]

Summary.

Unilateral leg vein infusion of 2 6 mg/min of aminophylline (theophyl lamine) or of 2 0 mg/min of theophylline resulted in excess diuresis on the infused side in hens This suggested a tubular point of attack

Pre-treatment with 50 mg/kg of probenecid did not affect the diuretic response to aminophylline, nor did a unilateral leg vein infusion of *1 mg/min* of probenecid cause any assymmetry in an established diuretic response to systemic aminophylline This probenecid dose was about optimal for unilateral depression of phenol red excretion

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[4] Although caffeine does not dissociate as an acid a unilateral leg vein infusion of 4 mg/min for 15 minutes resulted in ipsilateral excess of diuresis in one experiment but the over all response was considerably smaller than with theophylline at 2 mg/min No effect was observed with 2 mg/min caffeine infusion in one trial

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Interference of Reserpine with the Diuretic Action of Theophylline and Hydrochlorothiazide on the Chicken

By

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(Received September 16 1961)

It was fortuitously observed that a chicken having received reserpine on the previous day did not respond to a normally diuretic dose of aminophylline This unexpected finding led to a study of the diuretic activity of aminophylline (= theophyllamine), theophylline, hydrochlorothiazide, mercaptomerin and acetazolamide in both normal and reserpinised chicken

Methods.

The chickens and procedures were as described previously (NECHAY & SANNER 1961)

Serpedin ® (Pharmacia), supplied in ampoules containing 2 5 mg/ml reserpine was given generally as such, intramuscularly or intravenously In a few instances it was diluted with water to a concentration of 1 5 or 0 75 mg/ml for leg vein infusion When infused at any of these concentra tions into chicken plasma or saline *in vitro* reserpine was precipitated immediately after the solution had left the needle, as observed through a dissection microscope Thus reserpine probably also precipitates *in vivo* upon administration forming a depot in the muscle or crystals to be trapped in capillaries Since the absorption from such depots could be expected to vary greatly the doses given probably did not accurately reflect the true exposure of the site of action to reserpine In general the

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degree of sedation varied considerably among individuals and thus showed a strikingly variable degree of reserpinisation from a given dose Occasionally birds that received as much as 1 mg/kg reserpine exhibited no depression at all, with no altered response to hydrochlorothiazide or theophylline Some such non sedated birds were discarded before the diuresis experiment

Diuretics were given intravenously in 0 9% saline solutions at volumes of 1 ml/kg but theophylline was used at a concentration of 8 mg/ml

The renal clearance of polyethylene glycol (m v 4000) = macrogol 4000, infused into the wing vein at 30 mg/min (125 mg/ml saline), was used as a measure of glomerular filtration rate (HYDÉN & KNUTSON 1959, HYDÉN 1955) Blood samples were drawn from the other wing vein at the middle of urine collection periods through a polyethylene catheter

Results are reported in terms of means and their standard errors Figures in parenthesis in the tables indicate number of determinations when different from the number of experiments cited Points on the graphs correspond to the middle of urine collection periods

Results.

A *Effects of Reserpine itself on Urine and Cl Output*

After 1 mg/kg reserpine, 20 minute urine samples were collected for 13, 10 and 7 hours in 3 different hens The first two received the drug into the pectoral muscle and the third one into the wing vein Throughout the whole period the urine flows and Cl excretion rates remained within the range of values in the control animals

The comparison of control electrolyte and urine output in normal and reserpinised birds of table 1 and 2 (reserpine having been given many hours before) revealed no apparent differences except for the tendency of K^+ excretion to be somewhat higher in the reserpine treated groups In the few experiments in which glomerular filtration rates were measured, it seemed to be somewhat depressed by reserpine pretreatment

B *Single Wing Vein Doses of Diuretics*

In tables 1 and 2 are compared the response to four different diuretics in normal and reserpine treated birds

Reserpinisation markedly diminished the diuretic effectiveness of *hydrochlorothiazide* The most striking reduction occurred in Cl excretion, which was insufficient to match cation output Sodium excretion was reduced less markedly, but K^+ values remained closer to those of the control group The glomerular filtration rate possibly fell somewhat in reserpinised birds as well as in controls

Reserpine reduced dramatically the diuretic response to 10 mg/kg or 20 mg/kg *theophylline* The Cl^- and Na^+ outputs were almost completely

inhibited Similarly to what occurred with hydrochlorothiazide, K^+ excretion rate was reduced less than that of other ions

Generally, reserpine inhibited the excitement usually occurring during theophylline infusion

Mercaptomerin was about equally effective in normal and reserpine treated groups

In reserpinised birds the output of Na^+ caused by *acetazolamide* was perhaps greater than that of normal controls, but K^+ excretion and urine flow were less

C *Systemic Infusion of Hydrochlorothiazide*

In normal birds the maximum diuretic response to a wing vein infusion of 0 5 mg/min hydrochlorothiazide was reached in about 30 minutes In chickens receiving 1 mg/kg reserpine into the breast muscle 16 to 22 hours before, the effect of hydrochlorothiazide infusion was somewhat delayed, the outputs of Cl , Na^+ and water were more depressed than that of K^+ Even after 60 minutes of infusion the overall response was considerably less than in the control group (fig 1)

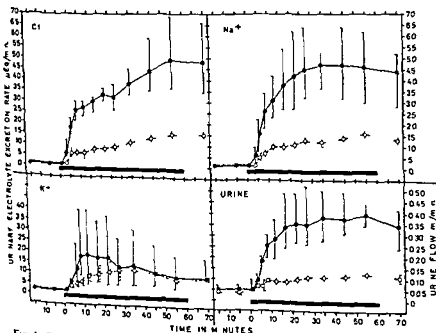


Fig 1 Diuret c response to hyd och ... pre treated with ... values for 3 nor ... Vertical lines and

Table 1.

Effects of some diuretics on the urinary electrolyte excretion in normal chicken

No of Expts Av wt	Time	Rate of urinary excretion					PEG clearance
		Cl⁻ (all expts)	Cl *	Na^+	K^+	Urine flow	
	min	μeq /min	μeq /min	μeq /min	μeq /min	μl/min	ml/min
8	(−20)–(−10)	1	1 (5)	2 (5)	2 (5)	68 ± 8	4 7 (3)
	(−10)–0	1	1 (5)	1 (5)	2 (5)	67 ± 12	5 4 (3)
2 7 kg		*Hydrochlorothiazide* 2 mg/kg wing vein					
	0–20	38 ± 4 2	35 ± 6 6 (5)	25 ± 2 2 (5)	13 ± 4 2 (5)	320 ± 28	4 8 (3)
	20–40	36 ± 3 7 (7)	36 ± 5 3 (5)	27 ± 5 6 (5)	11 ± 3 9 (5)	271 ± 21 (7)	4 5 (3)
	40–60	28 ± 2 9 (7)	27 ± 5 1 (5)	22 ± 4 0 (5)	7 ± 1 7 (5)	196 ± 22 (7)	4 5 (3)
4	(−20)–(−10)	1		–	–	48 ± 6	–
	(−10)–0	1	–	–	–	41 ± 6	–
3 3 kg		*Theophylline* 10 mg/kg wing vein					
	0–5	22 ± 4 8		–	–	221 ± 35	–
	5–10	27 ± 6 6	–		–	326 ± 99	–
	10–15	24 ± 7 4	–	–	–	274 ± 121	–
	15–20	21 ± 6 6	–	–		300 ± 141	–
	20–30	19 ± 5 5	–	–	–	232 ± 102	–
	30–40	19 ± 4 3	–		–	216 ± 74	–
	40–50	16 ± 4 4	–	–	–	180 ± 60	–
	50–60	14 ± 3 9	–	–	–	159 ± 52	–

	Period						
3	(−20)-(−10)	1	1 (2)	2 (2)	2 (2)	40	–
2.9 kg	(−10)-0	1	1 (2)	2 (2)	2 (2)	38	– *
	Theophylline 20 mg/kg wing vein						
	0-20	59	74 (2)	64 (2)	17 (2)	870	–
	20-40	93	134 (2)	108 (2)	11 (2)	1293	–
	40-60	40	57 (2)	64 (2)	6 (2)	780	–
6	(−20)-(−10)	1	1 (4)	2 (4)	2 (4)	56 ± 7	–
2.7 kg	(−10)-0	1	1 (4)	2 (4)	2 (4)	55 ± 8	–
	Hg 3 mg/kg wing vein as *mercaptomerin*						
	0-20	9 ± 3.7	7 ± 3.8 (4)	8 ± 5.5 (4)	3 ± 0.4 (4)	214 ± 73	–
	20-40	35 ± 12.1	32 ± 17.7 (4)	36 ± 22.5 (4)	3 ± 0.3 (4)	662 ± 236	–
	40-60	48 ± 8.4	37 ± 6.1 (4)	32 ± 6.8 (4)	2 (4)	526 ± 96	–
	60-80	17 ± 2.8	14 ± 3.0 (4)	10 ± 2.2 (4)	2 (4)	173 ± 25	–
	80-100	4 (2)	1 (1)	1 (1)	1 (1)	45 (2)	–
3	(−20)-(−10)	–	–	2	2	117	–
2.8 kg	(−10)-0	–	–	2	2	107	–
	Acetazolamide 10 mg/kg wing vein						
	0-20	–	–	8	26	279	–
	20-40	–	–	15	27	575	–
	40-60	–	–	12	25	434	–
	60-80	–	–	6 (2)	18 (2)	244 (2)	–
	80-100	–	–	3 (2)	22 (2)	137 (2)	–

* Cl^- excretion rate in experiments in which Na^+ and K^+ were also determined

Table 2.

Effects of some diuretics on the urinary electrolyte output in chickens pre treated with 1 mg/kg reserpine into the pectoral muscle at −16 to −22 hours *

No of expts Av wt	Time	Rate of urinary excretion					PEG clearance
		Cl⁻ (all expts)	Cl⁻**	Na⁺	K⁺	Urine flow	
	min	µeq /min	µeq /min	µeq /min	µeq /min	µl/min	ml/min
10	(−20)–(−10)	2	2 (4)	2 (4)	3 ±0 3 (4)	56 ± 6	3 7 (3)
	(−10)–0	2	3 (4)	2 (4)	4 ±1 0 (4)	68 ± 8	4 0 (3)
		Hydrochlorothiazide 2 mg/kg wing vein					
2 5 kg	0–20	10 ± 4 0	8 ± 2 9 (4)	16 ± 3 8 (4)	9 ± 2 8 (4)	*107* ± *14*	4 1 (3)
	20–40	6 ± 2 3 (7)	8 (3)	18 (3)	8 (3)	101 ± 16 (7)	3 5 (3)
	40–60	6 ± 2 3 (7)	8 (3)	11 (3)	7 (3)	83 ± 15 (7)	3 0 (3)
10	(−20)–(−10)	1	1	2 (4)	5 ± 1 0 (4)	47 ± 6 (8)	–
	(−10)–0	1	1	2 (4)	4 ± 1 0 (4)	51 ± 8 (9)	–
		Theophylline 10 mg/kg wing vein					
2 7 kg	0–10	3 ± 1 2	1	4 ± 1 8 (4)	10 ± 3 6 (4)	77 ± 13 (9)	–
	10 20	2	1	4 ± 1 8 (4)	11 ± 4 1 (4)	84 ± 14 (9)	–
	20–30	1	1	3 ± 1 5 (4)	12 ± 3 5 (4)	62 ± 9 (9)	–
	30–40	1	1	3 ± 1 1 (4)	8 ± 1 8 (4)	56 ± 8 (9)	–
	40–50	1	1	2 (4)	7 ± 1 5 (4)	54 ± 8 (9)	–
	50–60	1	1	2 (4)	6 ± 0 9 (4)	48 ± 6 (9)	–

8	(20) (10)	1	1	2 (2)	5 (2)	58 ± 14 (7)	–
2.5 kg	(10)–0	1	1	2 (2)	4 (2)	52 ± 9 (7)	–
	Theophylline 20 mg/kg wing vein						
	0–10	2	1	1 (2)	9 (2)	78 ± 21 (7)	–
	10–20	2 (6)	1	3 (2)	9 (2)	102 ± 40 (6)	–
	20–40	1 (6)	1	3 (2)	8 (2)	84 ± 26 (6)	–
	40–60	1 (6)	1	3 (2)	10 (2)	86 ± 18 (6)	–
8	(−20) (−10)	1	1 (2)	4 (2)	3 (2)	78 ± 20 (7)	–
2.7 kg	(−10)–0	1	1 (2)	4 (2)	3 (2)	70 ± 20 (7)	–
	Hg 3 mg/kg wing vein as *mercaptomerin*						
	0–20	2	2 (2)	6 (2)	5 (2)	174 ± 67 (7)	–
	20–40	36 ± 9.4	21 (2)	31 (2)	2 (2)	564 ± 138 (7)	–
	40–60	55 ± 9.3	62 (2)	46 (2)	2 (2)	475 ± 120 (7)	–
	60–80	31 ± 6.4 (7)	31 (2)	24 (2)	2 (2)	239 ± 63 (7)	–
	80–100	19 ± 6.0 (7)	33 (2)	27 (2)	2 (2)	145 ± 40 (7)	–
3	(−20) (−10)	–	–	1	2	151	–
2.5 kg	(−10)–0	–	–	1	2	134	–
	Acetazolamide 10 mg/kg wing vein						
	0–20	–	–	17	13	383	–
	20–40	–	–	19	7	308	–
	40–60	–		20	3	283	–
	60–80	–	–	14	3	168	–
	80–100	–	–	11	4	115	–

* One bird in the 20 mg/kg theophylline group received 1 mg/kg into the wing vein at −6 hours

** Cl^- excretion rate in experiments in which Na^+ and K^+ were also determined

In two other experiments with non-reserpinised birds 1 25 mg/min hydrochlorothiazide infusion for one hour resulted in peak Cl^- excretions of 60 and 110 μeq /min , after a further 15 mg/kg given as a single intra venous dose, the flow and Cl^- output did not increase any further Un fortunately no Na^+ determinations were done With the same hydrochlo rothiazide schedule tested on one reserpinised bird, Cl^- excretion reached 15 μeq /min , Na^+ 30 μeq /min and K^+ 5 μeq /min at the end of the infusion The subsequent single dose increased Cl^- excretion to 30 μeq / min and Na^+ excretion to 50 μeq /min , but K^+ excretion remained at 4 μeq /min Thus even enormous doses of hydrochlorothiazide were counteracted by reserpine

D *Systemic Infusion of Theophylline*

0 1–0 5 mg/kg reserpine given into one leg vein or 1 mg/kg reserpine given into the pectoral muscle 16 to 21 hours beforehand inhibited the diuretic effect bilaterally of 2 mg/min theophylline given over 15 minutes into the wing vein (fig 2) The Cl^- excretion rate due to theophylline given 10–21 days after 1 mg/kg reserpine into the pectoral muscle was still somewhat less than that of the control group, but this might not have been significant (fig 2) It may be seen, incidentally, that theophylline infused at 2 mg/min for 15 minutes into the wing vein of normal birds resulted in considerably smaller Cl^- output from both kidneys together than that caused by the same infusion directly into the renal portal circulation (cf fig 2 and 3A)

E *Unilateral Leg Vein Infusion of Theophylline or Aminophylline*

A unilateral leg vein infusion of 2 0 mg/min theophylline for 15 minutes, which normally resulted in a clearcut ipsilateral excess diuresis (fig 3 A modified from NECHAY & SANNER 1961), produced no significant diuretic effect on either side in birds pre treated with 1 mg/kg reserpine into the breast muscle 16-22 hours before (fig 3 B) The mean values for 6 control and 4 reserpinised birds are given for urinary flow, Cl and PAH excretion measured at each 2 5 minute collection period Reserpine also prevented the large bilaterally symmetrical fluctuations in PAH output seen in the control group Fig 3 B does not, however, include figures for one bird in which reserpine did not result in sedation and did not abolish the response to theophylline in terms of Cl^- (peak 30 μeq /min), urine (peak 0 2 ml/min) and PAH excretion pattern

In two controls and in one reserpinised bird (not shown), Cl excretion and urine flow remained unchanged during a control infusion of saline into the leg vein

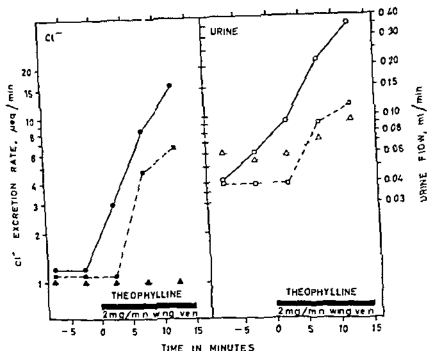


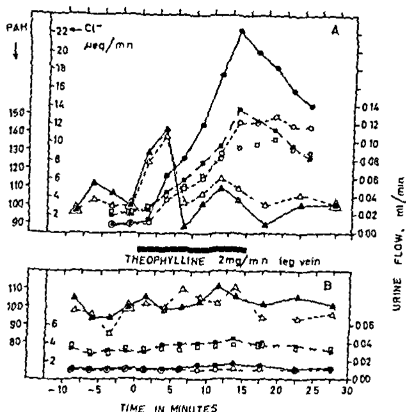
Fig 2 Effects of theophylline infusion on Cl excretion and urine flow in normal and reserpinised hens
●——● and ○——○ 5 normal birds ▲------▲ and △-- --△ 4 birds reserpinised at 16 to −21 *hours* Of these 3 birds were injected with 0 1 mg/kg 0 33 mg/kg and 0 5 mg/kg reserpine into one leg vein One was given 1 mg/kg reserpine into the pectoral muscle The flow values refer to two of these birds, ■ ■ and □ □ 4 birds reserpinised 10–21 *days* before with 1 mg/kg into the pectoral muscle

A similar series of experiments in 8 normal and 4 reserpinised hens was conducted with a unilateral leg-vein infusion of aminophylline at 2 6 mg/min (equivalent to 2 0 mg/min theophylline) for 15 minutes Only urine flow and Cl were determined

In this series also, reserpine inhibited the diuretic response (not shown), whereas the control group responded with an ipsilateral excess diuresis

F *Attempts to reserpinise one Kidney*

One bird, given 0 1 mg/kg reserpine unilaterally into the leg muscle 18 hours before, responded with a dramatic excess of urine and Cl⁻ output on the opposite side after a single wing-vein dose of 20 mg/kg theophylline As far as could be determined, the experiment was technically sound However, in two other experiments, one performed by the same procedure and another in which the dose of reserpine was doubled, good and about equal responses to theophylline were shown by the two kidneys Further, the reproduction of unilateral lack of diuresis was unsuccessful



at 1 mg/kg into the pectoral muscle 16–22 hours before PAH was infused into the wing vein at a rate of 0 2 mg/min throughout PAH values are given as percentages of the excretion rates in the periods just preceding theophylline infusion

in 11 other experiments in which the design was changed so as to infuse 25 to 500 µg/kg reserpine into one leg vein over 30 minutes 2 to 21 hours before theophylline was given Most of these birds responded normally to theophylline, except those in the upper dose and time ranges of reserpine treatment, when the effect had a tendency to be markedly reduced on both sides

In another type of experiment the parallel excretion of Cl and urine from both kidneys, induced by a wing-vein dose of 27 mg/kg aminophylline given 30 minutes previously, was not unilaterally altered upon leg-vein infusion of reserpine at 0 17 mg/min for 15 minutes

Discussion.

We have no satisfactory interpretation for the observed effects of reserpine The most obvious explanation, that the diuretic response to theophylline or hydrochlorothiazide was suppressed because of lowered

filtration rate, seems unlikely, since the effect of a mercurial diuretic was to produce virtually no change In normal birds theophylline at 20 mg/kg resulted in more diuresis than the dose of mercaptomerin used in this study, but reserpinisation suppressed the effect of theophylline but not that due to mercaptomerin

There are other possible explanations, but further work is needed to make a choice among them

The CNS-excitation and the large swings (of probably smooth muscle origin) in PAH excretion caused by theophylline were also prevented by reserpinisation Further work is planned to test the possibility that all the different effects of theophylline are blocked by reserpine

Summary.

Reserpine 1 mg/kg greatly reduced the diuretic effect of 2 mg/kg hydro chlorothiazide given systemically to the chicken, compared with normal controls The excretion of Cl^- was reduced to a greater extent than that of Na^+, but K^+ output remained close to controls Cl excretion was insufficient to match cation output Urine flow was markedly reduced Also the diuretic response to infusion of 30 mg hydrochlorothiazide over 1 hour was greatly reduced

Reserpine 1 mg/kg almost completely inhibited the Cl^- and Na^+ output due to 10–30 mg/kg theophylline As with hydrochlorothiazide, K^+ excretion was reduced less than that of other ions Urine flow was markedly reduced

Acetazolamide, 10 mg/kg, given to reserpine-treated birds resulted in a slightly reduced response in urine flow and K^+ excretion, whereas Na^+ excretion was, if anything increased Mercury 3 mg/kg as mercaptomerin was about equally effective in reserpine-treated birds and controls

Attempts to reserpinise only one kidney from the ipsilateral leg were unsuccessful except in one bird

The most obvious explanation that the reserpine effect on diuretic activity is due to reduced glomerular filtration rate, seems unlikely, since a mercurial diuretic was unaffected

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Determination of Lindane in Milk and Fat

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Lindane (the γ isomer of hexachlorocyclohexane) is one of the most commonly used insecticides of the chlorinated hydrocarbon type Careless use of its preparations has not infrequently caused acute poisoning of both human beings and livestock These chlorinated hydrocarbons accumulate in the fatty phase of the organism, so that the chemical detection and measurement of lindane is best performed on fatty tissue Its use in agriculture and veterinary medicine may lead to the presence of lindane residues in milk and slaughterhouse products A specific method for determining lindane in milk and fats is therefore needed

Various methods have been proposed for determining small amounts of lindane A comparison of them, with an appended comprehensive bibliography, was published by HEINISCH (1959), among others The colorimetric analysis of SCHECHTER & HORNSTEIN (1952) is one of the most specific and sensitive methods Moreover, it has the advantage that, apart from the spectrophotometer, it involves use of no particularly expensive apparatus

When using this method for determining lindane in fat or milk, we meet with the same problem as with all other analytical methods, namely that of concentrating the lindane and isolating it from interfering compounds

GYRISCO *et al* (1959), by combining a method by DAVIDOW (1950) for isolating chlorphenothane from fats and one devised by TUFTS *et al* (1950) for ultraviolet determination of lindane in milk and fats, evolved a method of purification that rendered lindane in milk determinable by the procedure of Schechter Hornstein

WOOD (1960) describes a similar method of isolation This, as well as the one mentioned above, are based on the fact that lindane is not attacked by a mixture of fuming and concentrated sulphuric acids

McKINLEY & MAHON (1959) MILLS (1959) and STORHERR & MILLS (1960) have described purifying processes suitable for subsequent qualitative and semiquantitative paper-chromatographic analyses of insecticides The methods are combinations of more or less modified single procedures previously described by others

All these methods of isolation have several features in common The procedure described below is based on the last-mentioned ones, which omit the disagreeable extractions with fuming and concentrated sulphuric acids It is also useful for determining a number of insecticides that cannot stand up to treatment with the strong acids

Principle of Analysis.

Combined extraction of lindane and fat Lindane is extracted together with the butterfat by a mixture of ether and light petroleum, after the proteins and mineral complexes of the milk have been precipitated with alcohol and potassium oxalate The different phases are easily separated without centrifugation The organic phase is washed with water and evaporated, after drying with sodium sulphate, direct on a boiling water bath (*Re* 1)

Acetonitrile partition Many insecticides can be extracted from light petroleum solution by acetonitrile (JONES & RIDDICK 1952), the main portion of the fat remaining in the petroleum phase This principle is employed to separate lindane from fat (*Re* 2)

By diluting the acetonitrile phase with water and extracting again with light petroleum, the insecticide is returned to the latter

Distillation The petroleum solution is evaporated, and the residue is dissolved in glacial acetic acid Volatile compounds, which would interfere with the succeeding colorimetric analysis, are removed by distilling off part of the glacial acetic acid

Colorimetric Determination of Lindane by the method of Schechter & Hornstein

The principle of this method is that of transferring lindane to benzene [illegible]

the benzene vapours are forced into a tube containing nitrating acid, and the benzene is there converted into m dinitrobenzene After dilution, the m-dinitrobenzene is extracted with ether The ether is evaporated, and the residue is shaken with methyl-ethyl ketone and 40% KOH to give a violet-red colour The colour intensity, measured spectrophotometrically, is directly proportional to the lindane concentration

Procedure.

Apparatus All glass apparatus as designed by SCHECTER & HORNSTEIN (1952)

Electric heating mantle
Shaking machine
Beckmann spectrophotometer, model DU with 1 cm absorption cells

Chemicals·

Ethanol, 96%
Ethyl ether, peroxide free
Light petroleum, b p 30–45°C

of light petroleum in a 2 litre flask The flask is shaken at intervals, and after 2–3 hours (or after standing over night) the phases are separated in a 1 litre separating funnel To the petroleum phase are added 100 ml of water and 100 ml of concentrated HCl in a flask The aromatic nitro-compounds developing are reduced by adding zinc in small portions, with shaking while at the same time the flask is cooled under the tap After the disappearance of the yellow colour, the phases are separated The light petroleum is washed with 100 ml of water before being distilled The fraction boiling below 45° is used

Acetonitrile, Fluka A G, redist, b p 97–82°C or Merck's purum reagens
Sodium sulphate solution, saturated, c p
Sodium sulphate, anhydr, c p
Potassium oxalate solution, c p, 10%
Liquid paraffin
Silicone grease, Dow Corning Stopcock Grease
In addition reagents required for the Schechter Hornstein s lindane determination

A *Milk*

Combined extraction of lindane and fat To 100 ml of milk, thoroughly homogenised by vigorous shaking, are added 100 ml of 96% ethanol and 10 ml of 10% potassium oxalate in a 1000 ml separating funnel The mixture is shaken vigorously for 1 minute After addition of 50 ml ether, the shaking is continued for 1 minute Finally, 50 ml of light petroleum are added After shaking for another minute the mixture is left standing for 20 minutes, to separate the phases

The aqueous phase is submitted to another two extractions in the same way with 25 ml portions of ether and light petroleum 10 minutes on each occasion suffices for proper separation of the phases

The combined ether and light petroleum phases are washed with 100 ml of distilled water by vigorous shaking for 2 minutes in the separating funnel

After 15 minutes' standing, the wash water is discarded, and the organic phase is filtered into a 250 ml Erlenmeyer flask through a 2 cm thick layer of anhydrous Na_2SO_4, kept in place by a tuft of cotton wool in a crucible holder The separating funnel is washed once with 10 ml of ether, which is also passed through the Na_2SO_4 layer A glass bead is put into the E-flask to prevent bumping, and the light petroleum and ether solution is evaporated on a boiling water bath to a 5–10 ml volume

Acetonitrile partition The residue is transferred to a 250 ml separating funnel, and the E-flask is washed a few times with altogether 20 ml of light petroleum After adding 25 ml of acetonitrile the separating funnel is shaken for 1 minute (mechanically) The phases have separated sufficiently after 3–4 minutes The acetonitrile phase, which is the heavier one, is transferred to a 1000 ml separating funnel containing 500 ml of distilled water

The light petroleum phase is extracted three more times with 25 ml portions of acetonitrile, and the acetonitrile phases are combined in the large separating funnel To this are added 50 ml of saturated Na_2SO_4 solution and 100 ml of light petroleum, and the mixture is shaken thoroughly for about 2 minutes After 10 minutes the phases separate The light petroleum phase is washed twice with 100 ml portions of distilled water

The wash water is discarded, and the light petroleum phase is transferred *via* a 2 cm thick anhydrous Na_2SO_4 layer to a 250 ml E-flask The Na_2SO_4 layer is washed with 10 ml of light petroleum

Distillation The light petroleum is removed by passing a current of air through the solution, the flask being kept at 30° in a thermostat bath The passage of air is stopped as soon as all the light petroleum has disappeared The residue is then highly viscous It is removed with the aid of 10 ml of glacial acetic acid, and the solution is transferred to the reaction flask of Schechter-Hornstein's apparatus The E-flask is washed twice with altogether 10 ml of glacial acetic acid, which is also transferred to the reaction flask 3 g malonic acid are added to the solution The mixture is then boiled on an electric hot-plate, with the flask placed at such a distance from the hot plate that the acetic acid vapours condense almost at the joint of the flask After one hour of boiling the heating is increased, and the solution is evaporated to 10 ml After cooling, 1 g zinc powder is added, and the analysis is continued as described by SCHECHTER & HORNSTEIN, except that the joints are lubricated with silicone grease (*Re* 3), and the ether extract of m-dinitrobenzene is evaporated on a boiling water bath, as described by GEHRKE *et al* (1957)

B *Fat*

3 g fat are cut into small pieces and ground with 20 g anhydrous sodium sulphate in a porcelain mortar To the pasty mixture are added 50 ml of light petroleum in a flask After mechanical shaking for 5 minutes, the light petroleum solution is decanted, and the residue is extracted twice more in the same way with 50 ml of light petroleum The extracts are collected in a 250 ml flask, filtered and evaporated to about 30 ml on a boiling water bath (Longer evaporation will result in precipitation of some fat) The solution is transferred to a separating funnel, the flask is washed with 10 ml of light petroleum, and the solution is extracted with acetonitrile, as described for milk

Remarks on the Method

Re 1

The high fat content of the solution prevents loss of lindane when the light petroleum is evaporated from it Hence there is no reason to employ a milder process of evaporation at this stage Attempts to evaporate the solution without previous washing with water and subsequent drying with sodium sulphate were unsuccessful The washing serves to remove the alcohol and water mixture, which dissolves in the light petroleum solution If this part of the procedure is omitted, the solution will separate into two phases towards the end of the evaporation period, and it will take a long time to remove the solvents

The inconvenience of bumping and consequent losses were also found to be reduced when the light petroleum and ether phase had been washed

Re 2

BURCHFIELD & STORRS (1953) have shown that N,N-dimethylformamide is better than acetonitrile for extracting lindane from *n* hexane However our experiments with N,N dimethylformamide in place of acetonitrile showed that the former has the disadvantage of also dissolving more fat from the light petroleum phase

Re 3

SCHLECHTER & HORNSTEIN lubricated the joints of their special apparatus with phosphoric acid However, if any of this acid gets into the reaction flask, it will interfere with the analysis GEHRKE *et al* therefore recommend acetic acid or dichloracetic acid as more suitable [illegible]

[illegible] gave the same extinction values as in the usual tests

Table 1

Absorption Figures for Lindane Standards with (B) and without (A) Distillation of Acetic Acid

Lindane standards µg	E_{565} A	Mean	E_{565} B	Mean
0	0 031	0 034	0 038	0 034
0	0 036		0 030	
15	0 112	0 105	0 101	0 108
15	0 097		0 114	
30	0 185	0 183	0 174	0 185
30	0 180		0 196	
45	0 277	0 265	0 254	0 256
45	0 253		0 257	
60	0 358	0 347	0 349	0 346
60	0 336		0 342	
75	0 394	0 410	0 406	0 425
75	0 425		0 443	

Results

A series of tests, performed with standard lindane solutions to investigate whether lindane is lost when part of the glacial acetic acid is distilled off before transfer to the Schechter-Hornstein's apparatus gave the results recorded in table 1, the extinction values in column B are for samples of lindane in the form of 20 ml of acetic acid solutions submitted to distillation, as described under Procedure The extinction values for corresponding 10 ml lindane acetic acid solutions transferred direct to Schechter-Hornstein's apparatus without previous distillation are set out in column A

The table shows that such distillation does not necessitate allowing for any loss of lindane

Table 2

Recovery of Lindane Added to Milk

Lindane added µg	E_{565}	Mean	Lindane recovered µg	Recovery per cent
0	0 027	0 036	0	
0	0 045			
15	0 095	0 090	12 0	80
15	0 084			
30	0 168	0 175	27 5	92
30	0 182			
45	0 252	0 228	37 5	83
45	0 204			
60	0 281	0 291	50 0	83
60	0 300			
75	0 354	0 345	60 0	80
75	0 336			

Table 3

Recovery of Lindane Added to Omental Fat

Lindane added µg	E_{465}	Mean	Lindane recovered µg	Recovery [1] per cent
0	0 033	0 042	2 5	--
0	0 052			
15	0 102	0 104	14 5	80
15	0 106			
30	0 177	0 179	28 5	87
30	0 180			
45	0 242	0 257	43 5	91
45	0 272			
60	0 295	0 326	56 5	90
60	0 357			
75	0 428	0 422	74 5	96
75	0 416			

[1] Corrected for blank

The usefulness of the analytical method has been tested by adding known amounts of lindane to samples of milk and fat The procedure described above was followed, and the results are given in tables 2 and 3

Discussion.

Schechter Hornstein's analytical method is relatively specific for lindane However, various volatile aromatic compounds will also give coloured ketone solutions Traces of these are often contained in the solvents best suited for extraction and isolation of insecticides, even after previous purification, their presence can render the analytical results valueless The procedure employed here, of distilling off some of the glacial acetic acid before the addition of zinc, proved to be an efficient way of removing such trace amounts GEHRKE *et al* (1957) showed that small amounts of water in the reaction flask cause low values, the nitrating acid being thereby diluted Other volatile solvents may possibly have a similar disturbing effect For this reason also the described process of distillation seems to be justified

When poisoning with a chlorinated hydrocarbon insecticide is suspected one is occasionally at a loss as to the substance to be looked for. In a toxicological laboratory, paper chromatography will usually be employed as a suitable "screening test" The method of extraction and isolation described above will then have the advantage that, combined with a suitable partition-chromatographic purification (MILLS 1959; MCKINLEY & MAHON 1959), it is useful for a qualitative determination of most chlorinated hydrocarbon insecticides by paper chromatography If lindane proves to be the substance in question, this can be determined

quantitatively by continuing the procedure with a suitable measured portion of the purified extract

In a similar way to fat, though larger samples will generally be needed, other products, such as berries, fruit and vegetables, as well as toxicological material, such as gastric contents, can also be examined for the presence of lindane and its amount

Summary.

A method is described for extracting and isolating lindane from milk and fat and then a quantitative determination of lindane, by a modified Schechter-Hornstein's procedure

The recovery after addition of known amounts of lindane to milk and fat has been found to be a minimum of 80 per cent

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From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller, M D)

Hexosamine, Hydroxyproline and Calcium Levels in the Tunica Media of the Human Aorta and Pulmonary Artery

By

Sv. Bertelsen

(Received September 21, 1961)

In the aortic wall and the pulmonary artery, three layers can be distinguished, the tunica intima, the tunica media and the tunica adventitia

The tunica intima, whose thickness increases with age, is built up of a few thin elastic and collagenous fibres, a number of cells (fibroblasts) and an abundance of ground substance

The tunica media consists mainly of elastic tissue It is built up of concentric elastic membranes, in the spaces between the membranes are thin layers of ground substance, containing a great many collagen fibres and cells (fibroblasts and muscle cells) The ground substance as well as the collagen fibres increase with age, so that the elastic membranes become separated

The tunica adventitia is relatively thin and cannot be sharply distinguished from the surrounding connective tissue The most outward of the tunica media membranes serves as an external elastic membrane, from which numerous elastic fibres project, and there is a gradual transition from the tunica adventitia into the surrounding loose connective tissue with its fat-cells

BERTELSEN (1961), from a histochemical study, described alterations in the human aorta and pulmonary artery with age He found a considerable increase of calcium in the medial tissue of human aortae with age, whereas no deposition is seen in intimal tissue of normal gross appearance The arterial ground substance consists of protein along with acid and neutral mucopolysaccharides The acid mucopolysaccharides contain hexosamine, uronic acid and possibly sulphate, the neutral mucopolysaccharides contain hexosamine, neutral sugars and possibly neuraminic acid, but not uronic acid or sulphate In previous biochemical investigations

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BERTELSEN (1960) and BERTELSEN & MARCKER (1961) have shown an increase in both acid and neutral mucopolysaccharides with age; the acid fraction consists of sulphated and non-sulphated carbohydrates An increase in acid mucopolysaccharides, due to an increase in the sulphate-containing mucopolysaccharides was demonstrated. BERTELSEN & MARCKER (1961) also published a few preliminary hexosamine results.

This paper presents the results of hexosamine, hydroxyproline and calcium determinations on the tunica media of the human aorta and pulmonary artery during ageing.

Materials and Methods.

102 samples of human aorta and 86 samples of pulmonary artery were obtained fresh at autopsy from the Rigshospital and the Copenhagen Municipal Hospital. The ages of the individuals providing the samples ranged from birth to 93 years.

None of the persons had suffered from collagen or hypertensive diseases or diabetes mellitus, and none had been treated with hormones.

The gross appearance of the aortae was subjectively assessed by estimating the percentage surface of the fibrous plaques in relation to the total intimal surface of the vessels The degree of atheterosclerotic changes was expressed as follows,

0: normal gross appearance
1: 5% fibrous plaques (slight atherosclerosis)
2: 5% < fibrous plaques < 25% (moderate atherosclerosis)
3 25% < fibrous plaques < 50% (severe atherosclerosis)
4: fibrous plaques > 50% (extremely vigorous atheroschlerosis).

All the aortic samples were drawn from parts with normal gross appearance in the upper part of the thoracic aortae (2–3 intercostal arteries), and the pulmonary samples from the pulmonary trunk

The adventitia and the intima were carefully removed by hand, and the separated medial tissue was washed with distilled water.

The medial tissue was freeze-dried and afterwards extracted to constant weight with pure light petroleum and ether (distilled over sodium)

The hexosamine content was determined on 35 mg of dry, de-fatted medial tissue. The sample was weighed on a torsion balance and hydrolyzed in sealed ampoules for 15 hours at 100°C with 4 ml of 2 0 N hydrochloric acid on a glycerol bath The hydrolysate was filtered, and the hexosamine concentration was determined in 3 ml of the filtrate by BLIX's modification (1948) of ELSON & MORGAN's method (1933) The readings were made at 530 mμ on a Beckman spectrophotometer

The hydroxyproline content was determined on 40 mg dry, de-fatted medial tissue. After being cut into small pieces, the tissue was hydrolyzed in sealed ampoules for 24 hours at 100° with 4 ml of 6 0 N hydrochloric acid on a glycerol bath The hydroxyproline content was determined in the hydrolysate by MARTIN & AXELROD's modification (1955) of NEUMAN & LOGAN's method (1950) The spectrophotometric readings were made at 560 mμ

The calcium was determined on 100 µl of the hydrolysate from the hexosamine analysis by the method of PATTON & REEDER (1956) All analyses were made in duplicate

The hexosamine and hydroxyproline values were corrected for the calcium content in the tissue Apatite (3 $Ca_3(PO_4)_2CaCO_3$) is known to be the predominant mineral in vessels but the mineral was calculated only as $CaCO_3$

Results.

Fig 1 shows the hexosamine content in medial aortic tissue in terms of dry de fatted and calcium free weight During the first three decades, there is a regular increase, in the fourth decade a sudden increase occurs, from the fifth decade on, a small but regular increase takes place Fig. 1 also shows the marked increase of calcium content with age

Table 1 shows the hexosamine values, partly in dry, de fatted medial tissue and partly in dry, de fatted calcium free tissue There is a distinct difference between the hexosamine values in the two columns, especially after the age of forty, and the difference increases with age There is a significant change in hexosamine contents of groups 1 and 4 ($p < 0.01$) and groups 4 and 5 ($p < 0.01$) An increase in hexosamine content after

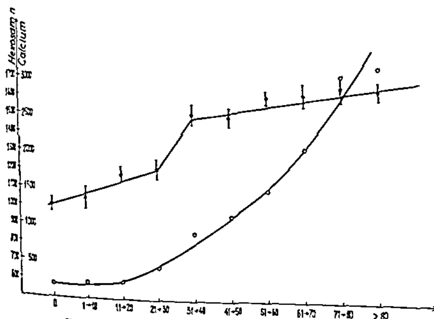


Fig. 1 Hexosamine and calcium in human medial aortic tissue at different ages. Abscissa age groups. Ordinate hexosamine content (●) in mg per 100 g dry de fatted decalcified tissue and calcium content (o) in 100 g dry de fatted tissue

Table 1

Hexosamine and calcium in medial aortic tissue at different ages

Group	Age	Number of samples	Hexosamine[1]			Calcium[2]	Percentage elevation of calcium
			dry, de fatted	dry, de fatted, decalcified			
1	0– 1	11	1009	1013	±34[3]	162	
2	1–10	4	1046	1051	±67	205	26 5
3	11–20	12	1170	1185	±41	225	9 8
4	21–30	7	1201	1215	±56	459	104 0
5	31–40	15	1480	1515	±55	929	102 3
6	41–50	12	1456	1500	±49	1168	25 7
7	51–60	10	1551	1606	±36	1510	29 2
8	61–70	10	1530	1618	±60	2086	38 1
9	71–80	9	1534	1659	±68	3052	46 3
10	>80	12	1506	1638	±50	3163	3 6

grand mean 1400

[1] mg per 100 g tissue
[2] mg per 100 g dry, de fatted tissue
[3] standard error of the mean

group 5 is seen distinctly in the column showing an allowance for mineral content, but there is no difference if the calcium concentration is ignored Table 1 further shows the calcium content and the percentage increase with age The percentage rise of calcium is greatest from groups 3 to 4 *and from groups 4 to 5 (more than 100 per cent)*

Fig 2 and table 2 show the amounts of hexosamine and calcium in the medial pulmonary tissue in terms of dry, de fatted, decalcified tissue There is a gradual increase in the hexosamine content with age, the difference between group 2 and group 10 is significant ($p < 0\ 001$) The hexosamine concentrations are lower in the pulmonary than in the aortic tissue The calcium values are low, and only after the age of sixty do they slightly increase

A comparison *is made, within the age groups 40–70 years, between the* hexosamine and calcium contents of vessels with and without atherosclerosis Of the 35 aortae, 15 show fibrous plaques (table 3) The average hexosamine contents of the two groups are identical The corresponding average calcium values show that the atherosclerotic samples contain no

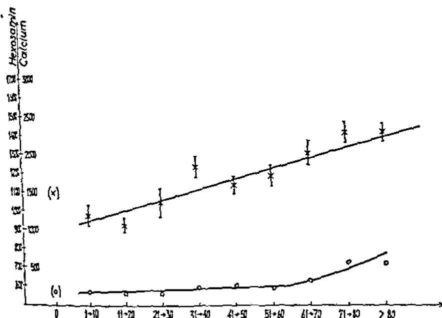


Fig. 2. *Hexosamine and calcium in human medial tissue from pulmonary artery at different ages.* Abscissa age groups Ordinate hexosamine content (x) in mg per 100 g dry, de fatted, decalcified tissue, and calcium content (o) in 100 g dry, de fatted tissue

Table 2

Hexosamine and calcium in medial tissue from pulmonary artery at different ages

Group	Age	Number of samples	Hexosamine[1]) dry, de fatted	Hexosamine[1]) dry, de-fatted, decalcified	Calcium[2])
(1	0– 1	1		1098 ±	194)
2	1–10	4		973 ±53[3])	188
3	11–20	7		924 ±36	152
4	21–30	7		1046 ±78	172
5	31–40	14		1229 ±52	243
6	41–50	12		1128 ±43	255
7	51–60	10		1170 ±54	242
8	61–70	10		1293 ±60	317
9	71–80	9		1400 ±56	557
10	>80	12		1401 ±43	538
				grand mean 1166	

[1]) mg per 100 g tissue
[2]) mg per 100 g dry, de fatted tissue
[3]) standard error of the mean

Table 1

Hexosamine and calcium in medial aortic tissue at different ages

Group	Age	Number of samples	Hexosamine[1]) dry, de fatted	Hexosamine[1]) dry de fatted, decalcified		Calcium[2])	Percentage elevation of calcium
1	0– 1	11	1009	1013	±34[3])	162	
2	1–10	4	1046	1051	±67	205	26 5
3	11–20	12	1170	1185	±41	225	9 8
4	21–30	7	1201	1215	±56	459	104 0
5	31–40	15	1480	1515	±55	929	102 3
6	41–50	12	1456	1500	±49	1168	25 7
7	51–60	10	1551	1606	±36	1510	29 2
8	61–70	10	1530	1618	±60	2086	38 1
9	71–80	9	1534	1659	±68	3052	46 3
10	>80	12	1506	1638	±50	3163	3 6

grand mean 1400

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[2]) mg per 100 g dry de fatted tissue
[3]) standard error of the mean

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A comparison is made, within the age groups 40–70 years, between the hexosamine and calcium contents of vessels with and without athero sclerosis Of the 35 aortae, 15 show fibrous plaques (table 3) The average hexosamine contents of the two groups are identical The corresponding average calcium values show that the atherosclerotic samples contain no

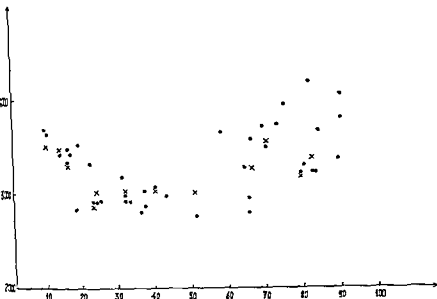


Fig 3 Hydroxyproline in medial tissue of human aorta (●) and pulmonary artery (x) Abscissa age Ordinate hydroxyproline in mg per 100 g dry, de fatted, decalcified tissue

Discussion

The first biochemical investigation on isolated human medial tissue was conducted by WEINHOUSE *et al* (1940) They found that the calcium content of medial aortic tissue increases with age (from 0 2% in children to about 3% of dry, de fatted tissue after the 5th–6th decades) The authors found that the increase of calcium in the media seems to indicate a normal physiological process having no relation with the degree of atherosclerosis of the intima

BIORNSSON (1942) showed that the logarithm of the calcium content of the aortic wall rises linearly with age The analyses were carried out on intimal medial tissue, and no agreement was found between the degree of atherosclerosis and the content of calcium.

In a biochemical investigation MYERS *et al* (1946) found a rise in the content of collagen after the fourth decade The analyses were performed on intimal medial tissue from the thoracic aortae

FABER (1949) found an increasing content of total sulphate with advancing years The percentage sulphate concentration is at least as high in senile aortae as in younger ones, and the author concluded that "the active part" of the vessel shows a rising percentage of sulphate with advancing age The author assumed that the total amount of sulphate in

Table 3

Hexosamine and calcium in medial tissue, partly in aortae with normal gross appearance or slight sclerosis (degree 0–1), and partly in atherosclerotic aortae (degree 2–4) the ages ranged from 40 to 70 years

none or slight atherosclerosis (0–1)			*moderate or severe atherosclerosis* (2–4)		
age	hexosamine[1]	calcium[2]	age	hexosamine[1]	calcium[2]
40	1563	866	41	1558	1938
40	1594	1003	46	1784	798
40	1577	1368	49	1536	1345
41	1399	616	49	1451	912
44	1534	502	50	1421	1186
46	1382	1368	57	1740	1300
47	1806	1915	58	1550	1140
47	1227	1003	63	1260	889
48	1342	1414	65	1622	2303
48	1569	1026	65	1667	1573
51	1528	1847	66	1555	2166
52	1665	1459	66	1626	2234
54	1693	844	67	1743	3648
55	1473	2212	69	1375	1003
58	1816	1140	70	1763	1938
59	1519	2166			
59	1574	1049			
59	1502	1938			
66	1652	1642			
69	1919	3466			
Mean 51	1567	1442	*Mean* 59	1577	1625

[1]) mg per 100 g dry de fatted decalcified tissue
[2]) mg per 100 g dry de fatted tissue

more calcium than what would be expected from the mean age of the group

On comparing areas of aortic medial tissue from single individuals, we found no difference between the calcium content of atherosclerotic areas and that of areas with normal gross appearance Table 4 shows duplicate analysis from 10 aortae

Further, the amount of calcium is no more in the abdominal aortic areas than in the thoracic aortae (table 5)

Fig 3 shows that the hydroxyproline content of medial aortic and pulmonary tissue varies little during life The values are calculated for calcium, and expressed in terms of dry, de fatted tissue Conversion to collagen is made by the use of the factor 7 46 There is a slight decrease in the second and third decades The amounts of hydroxyproline in the aortic tissue are of the same order as those in the pulmonary tissue

Table 5.

Medical calcium in various aortic sections
(mg per g dry, de fatted tissue)

	[1]				
	a	7 5		a	7 8
33 years	b	7 1	50 years	b	11 9
	c	8 2		c	12.1
	d	6 8		d	17 8
	a	7 1		a	11 9
41 years	b	6 8	55 years	b	11 0
	c	6 8		c	11 6
	d	6 4		d	12 3
	a	7 3		a	13 2
46 years	b	9 1	66 years	b	18 7
	c	8 9		c	20 1
	d	14 6		d	17 3
	a	6 2		a	13 7
49 years	b	9 0	76 years	b	14 8
	c	11 6		c	14 5
	d	8 4		d	14 4
	a	13 2		a	21 9
49 years	b	13 7	83 years	b	29 2
	c	14 1		c	28 7
	d	13 2		d	29 2

[1] a arch of the aorta
b uppermost part of the thoracic aorta
c lowest part of the thoracic aorta
d abdominal aorta

media) no changes in the hexosamine and acid-hydrolysable sulphate content with age In the pulmonary arteries they found no changes in the hexosamine concentration with age, whereas the acid-hydrolysable sulphate content was found to be higher in individuals aged 50–79 years than in younger subjects All the values were expressed in relation to not de fatted wet tissue

Buddecke (1958 a, b), in chemical analyses of extracts from thoracic aortic sections (intima-media) with normal gross appearance, found a relative decrease in elastic tissue with age, an increase in the hexosamine content and no change in the collagen with ageing All values were expressed in terms of dry de fatted weight Further, separate analyses showed an increase in the ratio glucosamine/galactosamine within the general increase of hexosamine

In a chemical analysis of atheroma-free sections (intima-media) from human aortae, Kanabrocki *et al* (1960) showed that the collagen level remained comparatively constant with advancing years (about 20% of dry tissue), whereas the calcium content gradually rose with age (0 07% in children to 0 84% in the age group 60–69 years, expressed in terms of dry tissue)

Table 4

Calcium in the medial aortic tissue (mg per g dry, de fatted tissue) corresponding to intimal areas with normal gross appearance (A) or with fibrous plaques (B) Duplicate analysis from ten aortae

Age	A	B
33 years	8 7 8 2	8 7 9 1
38 –	14 1 16 2	14 6 15 5
59 –	21 9 23 1	23 7 21 6
76 –	28 2 25 2	26 9 25 7
81 –	25 5 24 8	18 9 23 3
81 years	24 3 22 9	24 6 24 4
82 –	31 9 31 1	29 6 32 2
83 –	30 1 31 3	29 7 29 6
85	27 8 25 9	26 9 28 6
93 –	21 4 22 6	20 5 21 9

the tissue is derived from the sulphuric esters of carbohydrate (heparin, chondroitin-sulphuric acids) Further, FABER & LUND (1949) found that hypertension causes a greater rise in the calcium of aortic tissue (intima media) than would be expected from the age of the subject

KUZIN & GLADYSHEV (1950) determined the hexosamine content of human aortae on four samples derived from individuals 16 to 58 years of age The average hexosamine concentrations in intima, media and adventitia were 1 34, 1 23, and 0 66% of dry weight respectively

LANSING *et al* (1950), using 0 1 N–NaOH extracted elastin from human medial aortic tissue of various ages The authors mentioned that arterial elastic tissue develops an affinity for calcium with age, this calcium being deposited in or on the elastic tissue and consisting of apatite like crystals The results show that there is hardly any calcium in juvenile elastin (0 3–0 5%), but a steady increase in the calcium amount after 20 years of age, continuing to maximum levels in the fifth and sixth decades of life (6 7% calcium in dry elastin)

Further, LANSING and collaborators found that the change with age of the amount of calcium content in the medial elastin of the pulmonary artery is negligible compared with that for the aorta

BUCK (1951), too, found an increase in the percentage content of calcium with age The analysis was performed on intimal medial tissue, although the concentration of mineral in the atherosclerotic portions of aorta was significantly higher than that in normal portions, the increase associated with atherosclerosis is slight compared with that associated with ageing The ratio of calcium to phosphorus is similar to that found in bone

KIRK & DYRBYE (1956) found in a large group of aortae (intima

Table 5

Medical calcium in various aortic sections
(mg per g dry, de fatted tissue)

	[1]				
	a	7 5		a	7 8
33 years	b	7 1	50 years	b	11 9
	c	8 2		c	12 1
	d	6 8		d	17 8
	a	7 1		a	11 9
41 years	b	6 8	55 years	b	11 0
	c	6 8		c	11 6
	d	6 4		d	12 3
	a	7 3		a	13 2
46 years	b	9 1	66 years	b	18 7
	c	8 9		c	20 1
	d	14 6		d	17 3
	a	6 2		a	13 7
49 years	b	9 0	76 years	b	14 8
	c	11 6		c	14 5
	d	8 4		d	14 4
	a	13 2		a	21 9
49 years	b	13 7	83 years	b	29 2
	c	14 1		c	28 7
	d	13 2		d	29 2

[1] a arch of the aorta
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BUDDECKE (1958 a, b), in chemical analyses of extracts from thoracic aortic sections (intima media) with normal gross appearance, found a relative decrease in elastic tissue with age, an increase in the hexosamine content and no change in the collagen with ageing All values were expressed in terms of dry de fatted weight Further, separate analyses showed an increase in the ratio glucosamine/galactosamine within the general increase of hexosamine

In a chemical analysis of atheroma free sections (intima-media) from human aortae, KANABROCKI *et al* (1960) showed that the collagen level remained comparatively constant with advancing years (about 20% of dry tissue), whereas the calcium content gradually rose with age (0 07% in children to 0 84% in the age group 60–69 years, expressed in terms of dry tissue)

KAPLAN & MEYER (1960) isolated acid mucopolysaccharides from 33 aortae (intima media) and, among other things, characterized the com pounds by analysis They found no variation in the relative content of hexosamine, and the ratio glucosamine/galactosamine showed no signif icant change with age

The results presented here are in good accordance with previous biochemical and histochemical findings (BERTELSEN & JENSEN (1960), BERTELSEN (1960), BERTELSEN (1961), BERTELSEN & MARCKER 1961))

The hexosamine contents of the materials examined were higher than those found by KIRK & DYRBYE (1956) they expressed their hexosamine values in relation to both wet and dry calcareous tissue containing lipoid, which explains the lower concentrations of hexosamine found by them, especially in the elder individuals

The hexosamine values of KUZIN *et al* (1950) are in good accordance with the results presented here The results of BUDDECKE (1958 a, b) showed small concentrations of hexosamine compared with these, even though their results are expressed in terms of dry, de fatted weight KAPLAN *et al* (1960) did not mention the total hexosamine concentrations found by them

The hydroxyproline content found is in good accordance with the results of BUDDECKE (1958) and KANABROCKI (1960) whereas the collagen concentrations in the report of MYERS *et al* (1947) were lower This discrepancy may well be explained by the non specific methods used for collagen determination before 1950

The calcium values in the reports of WHINHOUSE *et al* (1940) BJORNS SON (1942), BUCK (1951) and LANSING (1950) agree with those reported here, whereas the calcium concentrations found by KANABROCKI (1960) were low compared with those found by the authors mentioned

The hexosamine values reported here represent the acid as well as the neutral carbohydrates In a previous investigation BERTELSEN (1960) showed that the increase in the hexosamine content with advancing age is attributable to an increase in the acid as well as in the neutral muco polysaccharides It was further shown that the percentage increase in hexosamine content of the acid fraction was considerably greater than that in the hexosamine content of the neutral fraction

Summary.

Hexosamine and hydroxyproline analyses have been performed on medial aortic and pulmonary tissue

All values are expressed in terms of dry de fatted decalcified tissue

In aorta as well as pulmonary artery an increase in hexosamine concentration was seen with age, whereas the hydroxyproline content was relatively constant

The hexosamine concentration in media was unrelated to the degree of sclerosis of the overlying intimal tissue

A big increase in calcium content of the medial aortic tissue was found, but the increase of calcium was shown also to be unrelated to the degree of atherosclerosis of the overlying intima

In the pulmonary artery the calcium content was low and relatively independent of age

Acknowledgement

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Acta pharmacol et toxicol 1961, 18, 370–378

From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller, M D)

The Action of Hydroaminacrine and some other Acridine Compounds on Isolated Guinea-Pig Ileum.

By

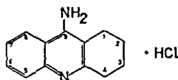
J. Jensen-Holm, K. Stubbe Teglbjerg and W. Hougs.

(Received September 7, 1961)

Hydroaminacrine (1,2,3,4-tetrahydro-9-amino-acridini chloridum) was prepared by ALBERT & BLEDHILL (1945) as one of a series of compounds whose bacteriostatic properties were tested by ALBERT *et al* (1945) (The constitutional formula is given below, the atoms numbered to conform with the Ring Index) The drug was found to possess no bacteriostatic action Large doses of hydroaminacrine may provoke convulsions in dogs These have not been described in man or other animals given corresponding doses SHAW & BENTLEY (1949) proposed to use hydroaminacrine as a morphine antagonist, since it counteracts the respiratory depression (STONE, MOON & SHAW 1961) It has, further, been found to stimulate the respiration of anaesthetised cats (SHAW 1960, HOUGS 1959) SHAW & BENTLEY (1953) demonstrated that the substance has a pronounced inhibitory effect on cholinesterase activity *in vitro* They found an inhibition of 50 % at a concentration of 10^{-7}M GERSHON & SHAW (1958) therefore attempted the use of hydroaminacrine partly as an antagonist to (+)-tubocurarine and to gallamonium and partly along with suxamethonium, whose action thereby was greatly extended Further, they observed that the drug intensified the action of acetylcholine on isolated intestine (guinea-pig, rat) and that it could produce contractions of isolated guinea-pig uterus, whereas it inhibited the action of added acetylcholine on isolated frog heart Moreover the drug was found to possess a weak antihistaminic action, though only at a concentration of 10^{-3}M or higher

Hydroaminacrine differs in chemical configuration from the commonly used anticholinesterases (e g physostigmine and neostigmine) This is one reason why we have examined the action of the drug on isolated guinea-

pig ileum and compared it with those of some other acridine compounds (acridine, 9 amino-acridine, euflavine and mepacrine) We have also studied the effect of hydroaminacrine in the presence of neuromuscular blocking agents ((+) tubocurarine, gallamonium, decamethonium and suxamethonium)



Technique

Male guinea pigs were killed by a blow on the neck and bled after decapitation

The ileum was removed and irrigated with the solution described below which was used in the cell A 4-5 cm long intestinal section, with no grossly visible Payr's plaques, was suspended in the cell with the heating device described by SECHER (1950) Touching the intestine was avoided The intestine was fixed above to a frontal recorder in equilibrium with 0 9 g giving suitably large deflections, which were recorded kymographically

Composition of the solution

NaCl	8 00 g
KCl	0 20 g
$CaCl_2$	0 20 g
$NaHCO_3$	1 22 g
$MgCl_2$ $6H_2O$	0 10 g
glucose	1 00 g
redistilled water to 1000 ml	

The solution was aerated with carboxygenium (Ph Dan 1948, Add 1956) containing 4% CO_2 and 96% O_2 On the basis of this CO_2 tension and in order to obtain a pH of 7 33 at a bath temperature of 37°C (±0° 2), the concentration of $NaHCO_3$ was calculated at 1 22 g/l by using the table of AHLGREEN (1930) The volume of fluid in the chamber was 25 ml in all the experiments The substances used in the experiments were added in solution by means of constriction pipettes in volumes that never exceeded 0 5 ml (50-500 µl) Change of fluid after each individual experimental period was performed by emptying and refilling with previously heated fluid three or four times through a glass tube leading from the bottom of the cell

In an attempt to decide whether hydroaminacrine as such possesses cholinergic activity we also used the fluid described above but without added glucose ...

... cosuginine (RIKER 1953))

I *The Substances Used*[1])

1) [illegible] ,2 3,4-tetrahydro 9 amino-acridine [illegible]

[illegible] rine chloride (B P) = 5 amino-acridine chloride B P = 9 amino-acridine chloride, Chem Abstract) Light & Co Ltd, England

3) Acridine, Th Schuchardt, Chem Fabrik, Gorlitz, Germany
4) Euflavine (Ph Dan 1948) (= acriflavine (WHO))
5) Mepacrine chloride (Ph Dan 1948)

II *Neuromuscular Blocking Agents*

1) Suxamethone iodide (curacit ®) (= succinylcholine iodide)
2) Decamethone iodide (synacur ®)
3) Gallamone iodide (NFN) (= flaxedil ®) (= gallamine triethiodide WHO)
4) *Tubucurarine chloride* (NFN)

III *Other Compounds*

1) Acetylcholine iodide Hoffmann La Roche and Co Ltd
2) *Histamine* chloride (Ph D)
3) *Mepyramine* maleate (Ph D)
4) Neostigmine bromide (Ph D)
5) Atropine sulfate (Ph D)

Results

A) *Action of Hydroaminacrine*

1) *Glucose containing fluid* Doses below 1 µg per 25 millilitres of total volume gave only slight or no contraction of the intestine Within the range of 1 to 10 µg (corresponding to 2×10^{-7} to 2×10^{-6}M) we found in 20 separate experiments that contraction increased with rising doses The maximum effect was attained within the range of 1–4 µg, larger doses had little additional effect, as shown in fig 1 the rate of contraction depended on the concentration, being slow at doses under 2 µg, with larger doses giving instantaneous contraction The external longitudinal muscles were the first to contract The time elapsing before peristalsis set in was shorter the higher the concentration After a very large dose the effects on the two layers of muscles occurred almost simultaneously

2) *Glucose free fluid* Addition of 1 µg (1 32 × 10^{-7}M) neostigmine bromide promptly caused pronounced contraction Addition of glucose, 25 mg (in 500 µl), to the fluid, to give the usual concentration of glucose, namely 1 g/l, produced no further changes With hydroaminacrine in doses of 1 to 10 µg no contraction occurred in glucose free solution, whereas instantaneous contraction and peristalsis were seen after addition of glucose, as already stated

[1]) *Numbers throughout conform with the Ring Index*

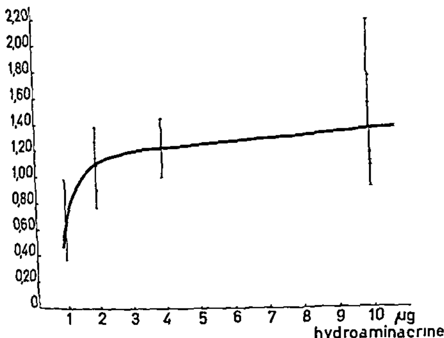


Fig 1 Isolated ileum from guinea pigs was suspended in a 25 ml cell in the modified Tyrode Locke s solution described. Different doses of hydroaminacrine were added, and the contractions were recorded kymographically The reactions were measured in millimetres and related to millimetre reactions obtained with 1 µg acetylcholine iodide *Ordinate* this ratio *Abscissa* µg hydroaminacrine chloride in 25 ml Above a dose of 5 µg (= 10^{-6} M) gave no appreciably increased contraction

B) *Hydroaminacrine in the Presence of Other Drugs*

1) *Pretreatment with atropine* (1–5 µg) for a few minutes prevented shortening of the intestine after addition of hydroaminacrine However, in our experiments atropine seemed not to prevent peristaltic movements This may have been due to too short a period of pretreatment

Addition of atropine after contraction reduced the deflection to the starting level or slightly below this

2) *Pretreatment with mepyramine* (5 µg), which prevents even fairly large doses of histamine from producing contraction, had no influence on the contraction caused by hydroaminacrine Yet large doses of mepyramine (100 µg) could temporarily reduce a deflection due to hydroaminacrine, whereas pretreatment with such large doses did not diminish the contraction due to hydroaminacrine

3) *Acetylcholine and hydroaminacrine* We found a synergistic action of acetylcholine on hydroaminacrine (of an additive nature in the experiments under review) In one experiment 1 µg acetylcholine gave a deflection of 32 mm and 10 µg hydroaminacrine gave one of 44 mm Simul-

taneous addition of 1 μg acetylcholine and 10 μg hydroaminacrine gave a deflection of about 73 mm

4) *Neostigmine and hydroaminacrine* These two drugs were also found to show synergism Thus, in one experiment a deflection of 50 mm was seen in response to 1 μg neostigmine and of 82 mm in response to 10 μ hydroaminacrine, whereas half of these doses together gave a deflection of 85 mm

C) *Effects of Other Acridines*

1) *Acridine* Doses ranging from 10 to 130 μg produced no contraction of the intestine Lower concentrations caused lenghthening However, pretreatment with acridine (32 μg) intensified the action of acetylcholine (1 μg)

2) 9-*Amino-acridine* Doses of 10 μg produced moderate contraction This, however, was brought about at a much slower rate than after the same dose of hydroaminacrine, indicating that 9-amino acridine has a much weaker action

3) *Euflavine*

10 μg gave a deflection of 33 mm
40 – – – – – 18 –
80 – – – – – 10 –

Pretreatment of the intestine with 80 μg intensified the action of 1 μg acetylcholine

4) *Mepacrine*

10–30 μg gave little contraction
50–100 μg relaxed the resting position
50–100 μg reduced reactions to hydroaminacrine
100 μg completely suppressed reaction to 10 μg hydro-aminacrine
50–100 μg reduced reactions to acetylcholine

D) *Interaction of Hydroaminacrine and some Neuromuscular Blocking Agents*

1) (+)-*Tubocurarine* (+) tubocurarine alone relaxed the intestine in doses from 5 to 20 mg This relaxation was intensified by hydroamina crine (10 μg) It may be added that 3 μg acetylcholine abolished the intestinal relaxation produced by 12 mg tubocurarine

2) *Gallamonium* 12 mg gallamonium effected slight contraction of the intestine, which was increased by adding hydroaminacrine

3) *Decamethonium* 400–600 μg decamethonium caused contraction of the same order as did 10 μg hydroaminacrine In one experiment we found that 300 μg decamethonium plus 5 μg hydroaminacrine gave a reaction corresponding to that obtained with 10 μg hydroaminacrine

4) *Suxamethonium* Doses of 10 mg produced contraction of the same order as did 1 µg acetylcholine Doses of 5 mg suxamethonium plus 5 µg hydroaminacrine caused the intestine to contract to the same extent as did 10 mg suxamethonium or 10 µg hydroaminacrine This indicates a synergistic action

Discussion

In the experiments conducted with isolated guinea pig ileum suspended in the Tyrode Locke s solution modified as stated addition of hydro aminacrine was shown to produce contraction of the intestine The nature of this was investigated in our experiments The contraction can evidently be explained by an anticholinesterase activity similar to that of physostigmine

Production of the contractions reported depends on among other things the presence of glucose in the solution This is evidence to suggest that hydroaminacrine in itself has no influence on the receptors like physostigmine (BURN 1952) but unlike neostigmine (RIKER 1953)

SHAW & BENTLEY (1953) studied the cholinesterase activity and found it to be inhibited 50% at a hydroaminacrine concentration of 10^{-7} M JENSEN HOLM (1959) by titrimetric estimation (JENSEN HOLM LAUSEN MILTHERS & MOLLER 1959) at a molar concentration of 4×10^{-7} found 70% inhibition (diluted whole blood 38°C acetylcholine as substrate at a concentration of 4×10^{-3} M) The concentrations used in the experi ments reported 1 10 µg hydroaminacrine per 25 millilitres correspond to 2×10^{-7} to 2×10^{-6} M On the basis of these numerical values we are probably justified in regarding the effect of hydroaminacrine as due solely to anticholinesterase action on the intestine

As stated we found a synergistic action of hydroaminacrine by neostig mine About ten times as much hydroaminacrine as neostigmine (by weight) is required to produce the same intestinal contraction Both drugs also provoke contraction of the circular muscle fibres (peristaltic movements) However with the equivalent doses mentioned peristalsis was released promptly by hydroaminacrine whereas with neostigmine there was an interval of a few seconds This suggests that neostigmine a quaternary ammonium compound diffuses at a slower rate through the intestinal wall

After having found a synergistic action between hydroaminacrine and acetylcholine we also studied the interaction of hydroaminacrine and atropine We noticed that pretreatment with atropine prevented reaction to hydroaminacrine Likewise a contraction caused by the drug could be

abolished by adding atropine These results are further evidence to suggest that the effect of hydroaminacrine is mediated by acetylcholine

To establish that hydroaminacrine does not liberate histamine, we have shown that pretreatment with mepyramine (5 μg) has no effect on the action of hydroaminacrine

Hydroaminacrine and Neuromuscular Blocking Agents

Four different neuromuscular blocking agents were used For their action on the intestine they may be divided into two groups one ((+) tubocurarine) relaxes the intestine (McINTYRE 1947), whereas the other (gallamonium, decamethonium and suxamethonium) brings about a more or less pronounced contraction of the intestine We invariably found that hydroaminacrine intensified these actions, having increased the relaxing effect of (+)-tubocurarine and acted synergistically with gallamonium, decamethonium and suxamethonium The finding of a weak contracting effect of gallamonium on isolated intestine is inconsistent with the results of experiments conducted by HOUGS & JOHANSEN (1957), in which no effect was noticed under similar experimental conditions

We cannot explain why (+) tubocurarine and gallamonium have opposite actions on the intestine The two drugs are commonly believed to possess identical mechanisms of action on striated muscle through neuromuscular transmission Note, however, that the concentrations required of these drugs to produce an effect on the intestine are considerably higher than those usually employed clinically Neither is it possible to explain why hydroaminacrine intensifies the relaxing effect of one drug and the contracting effect of the other Addition of acetylcholine in suitable doses can abolish the action of (+)-tubocurarine The concentration of acetylcholine brought about by hydroaminacrine thus seems not to be raised sufficiently

On the other hand, the synergistic action of decamethonium or suxamethonium on intestinal contraction can be explained by the acetylcholine-protecting action of hydroaminacrine

Hydroaminacrine, Acridine and Other Acridine Derivatives

As the chemical configuration of hydroaminacrine differs essentially from that of neostigmine, for example, we compared the drug with other acridines for their actions on isolated intestine, to discover if they act specifically on the sphincters

Acridine caused no contraction of the intestine, though it could intensify the action of acetylcholine (acridine is a weak anticholinesterase (AUGUSTINSSON 1948)) However, 9 amino-acridine produced contraction

in doses about ten times larger than those of hydroaminacrine In other words, it appears that the compound must contain an amino group to be able to cause contraction of intestinal muscles The drugs used can, however, give no information as to the significance of the location of the amino group

Euflavine with two amino groups, behaved somewhat differently At low concentrations it produced a contraction, which could be reduced by increasing the dose As stated above, acridine at low concentrations may have a relaxing effect on the intestine With euflavine, it may therefore be possible that at low concentrations the amino acridine action (contraction) predominates, whereas at higher concentrations the acridine action (relaxing) is the more pronounced

Similarly, it was seen, to an even more pronounced degree, in the experiments with mepacrine, that small doses gave slight contraction, whereas larger doses even relaxed the resting position, and also caused reactions due to acetylcholine or hydroaminacrine to be reduced With fairly large doses the action must therefore be of an atropine-like character

Summary.

The action of hydroaminacrine (1,2,3,4-tetra hydro 9 amino-acridine chloride) on isolated guinea pig ileum has been studied The drug produced contraction of a nature explainable on the basis of an anticholinesterase action corresponding to that provoked by physostigmine Hydroaminacrine as such had no effect on the receptors Atropine counteracted the action of hydroaminacrine, whereas mepyramine was found to be inactive

The action of the drug in the presence of neuromuscular blocking agents was also investigated It was found to intensify the actions of (+)-tubocurarine (relaxing) as well as gallamonium, decamethonium and suxamethonium (contracting)

Further, the actions of other acridines acridine, 9 amino acridine, euflavine and mepacrine, were examined Basically 9 amino acridine produced the same reactions as hydroaminacrine, but acridine, euflavine and mepacrine did not This suggests that the presence of an amino group and its location are both of fundamental importance for the effects studied

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The Cholinesterase Activity, alone and in the Presence of Inhibitors, at low Substrate Concentrations

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(Received November 13 1961)

It is well known that the values obtained analytically for cholinesterase activity depend on the concentration of acetylcholine employed The unspecific cholinesterase (= pseudocholinesterase = plasma cholinesterase = butyryl cholinesterase = ChE) shows an increase in activity with rising substrate concentration within the entire range of possible measurements whereas the specific cholinesterase (= "true" cholinesterase = erythrocyte cholinesterase = AChE) shows maximum hydrolytic activity between 10^{-2} and 10^{-3} M acetylcholine With both rising and falling substrate concentrations the values decrease (*vide* AUGUSTINSSON 1948 and others)

In determining any particular enzymatic activity it is therefore not only a matter of interest to know this at some fixed – usually a high – substrate concentration, but even more so for purposes of physiological and pharmacological understanding to know the activity at the acetylcholine concentration occurring *in vivo* Although the physiological concentration is unknown, we have tried here to estimate its order (see further under *discussion*) It is thought to lie close to 10^{-6} M at the motor end plate, a concentration, in other words, much lower than those usually employed during analysis (10^{-2}–10^{-3} M) Accordingly we find much lower activity values Further, in the presence of reversible inhibitors, used as competitors with acetylcholine, the degree of inhibition will become more and more pronounced the lower the substrate concentration used for the measurement This is a phenomenon well-known to enzymologists, but somewhat unheeded by pharmacologists and toxicologists In a paper by BERNHEIM & BERNHEIM (1936) it is plainly shown that morphine and ...creasing inhibition of [ChE] (measured as a pro-

longed time of hydrolysis) with decreasing amounts of acetylcholine used, though measures were made only at substrate concentrations down to about 9×10^{-4} M The same thing has been demonstrated by KUHN & SURLES (1938) (down to about 2×10^{-4} M *acetylcholine*), *cf* WRIGHT & SABINE (1943) BURGEN (1949) stressed that "Methods of estimation in common use greatly underestimate the physiological activity of the reversible cholinesterase inhibitors because of the competition with substrate" He established the truth of this view in experiments with physostigmine (though the lowest acetylcholine concentration was 3×10^{-3} M) AUGUSTINSSON (1948) also touches on the problem from the standpoint of enzyme kinetics, but without going into details about any pharmacological conclusions that might be drawn

To achieve a more thorough physiological knowledge (cf WURZEL 1960) and a more exact pharmacological and toxicological understanding of the enzymatic activity, we have aimed at studying cholinesterase activity at substrate concentrations approaching the phyciological (JENSEN HOLM 1960)[1])

To work at such low substrate concentrations it has been necessary to modify the analytical method in such a way that the normal hydrolytic process, which is of the first order, is altered so as to appear as a process of zero order, by keeping the substrate concentration at a constant level

Symbols used

AChE erythrocyte cholinesterase
ChE plasma cholinesterase
[AChE] Erythrocyte cholinesterase activity in µmol per minute per gram tissue
[ChE] plasma cholinesterase activity in µmol per minute per gram tissue
ACh acetylcholine
C_S substrate concentration
pS the negative logarithm of the substrate concentration C_S

General Methods

The basis of the method is that described by JENSEN-HOLM, LAUSEN, MILTHERS & MOLLER (1959), in which [ChE] was determined by automatic titration with Radiometer's "Titrator" and "Titrigraph"

1) In part in the form of a paper read before the Xth Scand Physiol Congress in Oslo 1960

The *activity*, which is a measure of the rate of hydrolysis, is estimated at 38°C and pH = 7 40, being expressed as µmol per minute per gram tissue or millilitre of blood It is derived from the difference between the slopes of the curve after and before addition of acetylcholine (i e total activity minus blank activity) Further, correction should usually be made for non-enzymatically caused substrate hydrolysis

Blank activity means spontaneous or indused, non-specific acid liberation or production, not altered by the possible presence of acetylcholine(JENSEN-HOLM *et al* 1959) Such acid production takes place in most tissue homogenates or blood dilutions It is sufficiently constant during the relatively few minutes of measurement Often, however, it is so large that it greatly exceeds the specific acid liberation (the real cholinesterase activity), especially when the substrate concentration is low By keeping the system anaerobic (bubbling nitrogen through it for 20 minutes and then maintaining an atmosphere of nitrogen in a half-closed system) the spontaneous, unspecific acid liberation can be not only greatly reduced, but also often converted into a fairly constant basic production As a blank activity that is either zero or – preferably – slightly base consuming is a technical prerequisite for measuring [ChE] it has been necessary to be able to adjust it to such suitable value For this purpose we have constructed an infusion apparatus for constant supply of acid (HCl) or base (NaOH) to the reaction mixture The infusion apparatus can contain up to six glass syringes of different lengths and volumes, from 1 to 20 ml The supply rate can be varied continuously by a conical gearing system

Non Enzymatically Determined Substrate Hydrolysis

At substrate concentrations above 10^{-4} M correction was made for the non enzymatically caused hydrolysis of acetylcholine At lower concentrations this was unnecessary, the error obtained by omitting such correction being practically *nil*, because the degree of non-enzymatically caused hydrolysis of substrate at the temperature and pH employed depends solely on the *amount* of substrate used (JENSEN-HOLM 1960, unpublished) In the given circumstances 0 015% of the amount of acetylcholine iodide present in the reaction mixture is split per minute

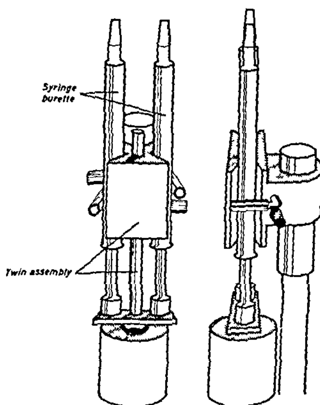
Example 0 1 g brain (guinea pig) in 100 ml of reaction mixture at a substrate concentration of 10^{-5} M gave a normal activity of 1 µmol/min /g, or 0 1 µmol/min /0 1 g The non-enzymatically determined substrate hydrolysis – 0 015% ∾ 0 00015 µmol/min The error due to omitting [illegible]

[illegible] µmol/min /0 1 g equivalent to 5 and 4 85%,

respectively, of the corresponding normal value, is of no significance in practice, particularly since the error lies within the limits of experimental accuracy

Measurement of Normal [ChF] at Low Substrate Concentrations

With substrate concentrations below 10^{-4} M, maintenance of a constant substrate concentration is required to permit measurement of the slope of the curve with sufficient accuracy This was done by replacing the single syringe burette belonging to the titrigraph by a *Twin Assembly DS 60083* carrying two simultaneously working Agla Syringe burettes instead of the previous one (The Twin Assembly was developed by JENSEN-HOLM in collaboration with *Radiometer*), see fig 1



Twin assembly for SBU 1
Type DS 60083

type DS 60083 which
by a common micro
The change of volume
r screw (corresponding
raph passes) is 500 µl

One syringe contains NaOH for titration and the other acetylcholine iodide of a strength equimolar in base content Assuming that the blank activity is adjusted to zero or nearly so, it is possible to maintain a constant substrate concentration for a sufficiently long time The titration curve then changes from a markedly curved course to one that is either rectilinear or slightly curved one way or the other The tangent indicates the slope at the starting point In this way a process of the first order is converted into one that appears to be of zero order

By using a sufficiently large total volume of reaction mixture (up to 250 ml) and thorough magnet stirring it was possible to work at substrate concentrations as low as 2×10^{-6} M

The concentration of the base and acetylcholine in the solutions in the Agla Syringes was usually 5–10 mM The strength of the base is determined by titrating with potassium hydrogen tartrate by means of the titrigraph either to a fixed "endpoint" or by plotting the titration curve

Results.

A Normal Cholinesterase Activity:

The results are shown for three different normal tissues, haemolysate, plasma (human) and guinea pig brain We worked with low tissue concentrations in a solution of 0 9% NaCl in freshly redistilled water

1 *Haemolysate from Human*

Three times washed and haemolysed erythrocytes were used at a concentration of 1/6% (e g 1 ml of 25% haemolysate to 150 ml of reaction mixture)

The results are shown in fig 2

The hydrolysis curve shows maximum hydrolysis at 10^{-3} M of substrate It runs a bell shaped course (cf AUGUSTINSSON 1948) Logarithmic values for the substrate concentration constitute the abscissa If the ordinate values are also expressed logarithmically (upper curve), the points of inflection must lie at pS = 4,0 – 3 8 and 1 9 – 1 7 (– = pK_1 and pK_2 (AUGUSTINSSON 1948)), and the logarithmic courses to the left and right of the points of inflection seem to be linear The numerical coefficients of regression are, however, not absolutely identical On the left the activity becomes 5 1 times lower with each ten fold reduction in substrate concentration on the right one the activity decreases 5 9 times with each ten fold rise in substrate concentration

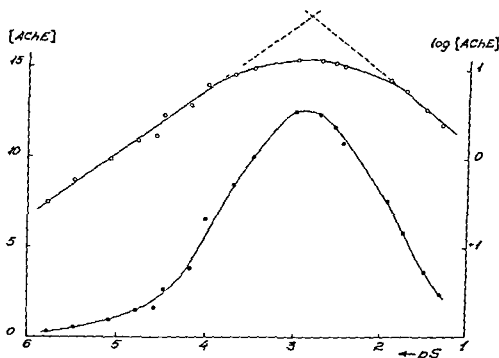


Fig 2 Erythrocyte cholinesterase
The *abscissa* is the negative logarithm of the substrate concentration (= pS)

maximum is at pS = 2 86

The fact that the logarithmic courses to the left and right of the points of inflection are linear seems of particular interest at low substrate concentrations, because this offers the possibility of extrapolating activity values at substrate concentrations so low that they do not allow of direct measurement

2 *Human Plasma*

Plasma from fresh heparinised blood was used (with the lowest heparin concentration possible) in the form of 2% plasma in 0 9% NaCl solution The results are shown in fig 3 As in fig 2, this chart illustrates the course of the values if both co-ordinates are expressed logarithmically (upper curve) The curve is rectilinear up to $pS = 4\,0 - 3\,8$, after which it bends, possibly at a point of inflection It can be calculated from the coefficients of regression for the linear course that the activity decreases 6 9 times with each ten-fold reduction in substrate concentration

Fig 3 Plasma Cholinesterase

Plasma from human blood (from the same sample as used for the analysis illustrated in fig 2)

[illegible]

ac [illegible]

cu [illegible]

so[illegible]e kind of point of inflection as in fig 2

3 *Homogenate of Guinea Pig Brain*

The cerebrum and diencephalon were homogenised at 0° in a homogeniser made by Measuring & Scientific Equipment Ltd (M S E Cat no 7700) The tissue concentration used was 1/4%

The results are shown in fig 4 The curve is seen to resemble that in fig 2 Within the range stated the curve runs a logarithmically linear course with a coefficient of regression corresponding to an activity that decreases 6 0 times with each ten fold reduction in substrate concentration

For all three tissues we have illustrated (the curves are not shown) the results on the basis of HOFSTEE's (1952) variation of MICHAELIS-MENTEN's (1913) equation by plotting the enzyme activity (ordinate) against the enzyme activity divided by the substrate concentration (abscissa) In no instance did this give straight lines (cf AUGUSTINSSON 1948, DIXON & WEBB 1958)

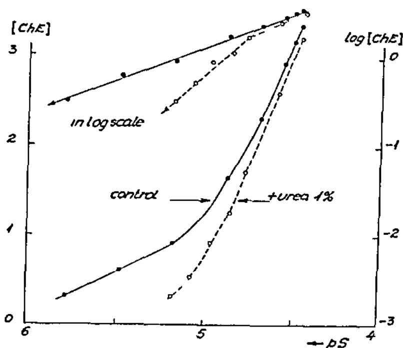


Fig 4 Brain cholinesterase from guinea pig

The unbroken curves

Lower *Ordinate* brain cholinesterase activity in µmol/min /g brain tissue
Abscissa negative logarithm of substrate concentration (− pS)

Upper *Ordinate* the same expressed logarithmically
Abscissa pS

The upper curve is seen to be linear within the range stated

The broken curves illustrate the conditions in the presence of *urea* at a concentration of 1%

The upper broken curve appears to be linear at pS − 4 6 (2 5 × 10 ⁵M)

B: [ChE] in the Presence of Some Inhibitors.

1 *Reversible Inhibitors* (see figs 5 and 6)

a) Physostigmine Salicylate

After adding 0 7 ml of 25% human haemolysate to a 0 9% NaCl solution in redistilled water and making up to a volume of 75 ml the mixture was incubated at 38° for 70 minutes with physostigmine salicylate at a concentration of 2.5×10^{-8} M The substrate concentrations used were those given in fig 5 Control values used were those obtained in a corresponding series of analyses without physostigmine The tangent of the curve plotted immediately after adding substrate has been taken as a measure of the total activity, corrected in the usual way for blank activity and non enzymatic substrate hydrolysis It is seen in fig 5 that the degree of inhibition rises with falling substrate concentration

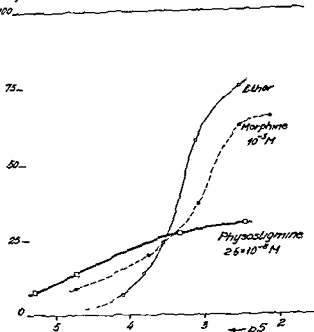


Fig 5
Relative residual activity after different inhibitors at various substrate concentrations

Abscissa negative logarithm of substrate concentration (– pS)
Ordinate The measured cholinesterase activity as percentage of the corresponding normal activity in the ab[illegible]

[illegible] physostigmine salicylate
[illegible] for 70 minutes at neutral reaction before
[illegible]
[illegible] concentration 10^{-3} M morphine chloride
Tissue [illegible]
[illegible]

b) Morphine Chloride[1]

1 ml 25% human haemolysate, diluted to a total volume of 25 or 50 ml, was incubated with morphine at a concentration of 10^{-3} M for 10 minutes at 38° Substrate was then added at different concentrations The relative

[1] The analyses were carried out by TH. JÓHANNESSON (in 1960) and kindly placed at the author's disposal for comparison with the remaining results (cf JÓHANNESSON 1962)

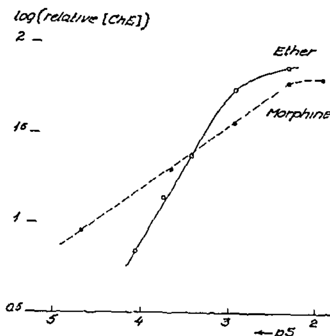


Fig 6 Results from fig 5

[ChE]/
ints of

activity displayed greater variation with falling substrate concentration than occurred with physostigmine

c) Ether

Incubation was performed with pure ether distilled over sodium stored with added KOH (Struer s Chem Lab) We used 1 ml of a 20% guinea pig brain homogenate diluted to a final volume of 75 ml After adjustment of the blank activity 2 ml of ether were added in a closed system with magnetic stirring Exactly 120 seconds later substrate was added in different amounts The actual concentration of ether was unknown As, however, the procedure was the same in all the analyses the ether concentrations may be assumed to have been very nearly identical and lower than 2 6% (v/v) [ChE] as a percentage of corresponding normal values showed a pronounced dependence on the substrate concentration employed

Fig 5 shows for all three inhibitors that the relative [ChE] decreases with falling substrate concentrations followed typical biological curves The lower the specificity of the inhibitor for the cholinesterase, the greater the dependence on changes in substrate concentration When we

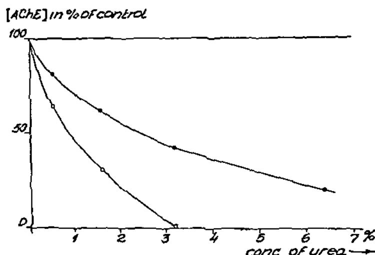


Fig 7 Human haemolysate plus urea

se activity

Lower curve = 8 3 × 10^{-6} M

Us ng the same urea concentration a definitely stronger inhibition is obtained at the lowest substrate concentration

If the ordinate is expressed logarithmically (not included here) both curves will run approximately rectilinear courses

express the ordinate values logarithmically (fig 6), we find that the curves for ether and morphine run linear courses to the left of the points of inflection *Hence there must be the possibility* of extrapolation to unanalysed values at substrate concentrations *lower than* those shown on the chart The same is possibly true of physostigmine, but the point of inflection at the concentration employed seems to lie at about 10^{-5} M Ach

d) Urea

Fig 7 shows the results *after incubation* with urea, which is a weak cholinesterase inhibitor Haemolysate at a low concentration was used as substrate tissue, and the two curves represent the values *obtained at* two different concentrations It is clear that the inhibition was most pronounced at the lowest concentration Each of the two curves is linear when the ordinates are expressed logarithmically

In fig 4 (guinea pig brain) the course is seen after incubation with 1% urea (lower curve) Each of the curves plotted is linear up to the point of inflection, provided both axes represent *logarithmic* values A curve

showing the remaining activity (after urea), expressed as percentage of the corresponding normal activity, must therefore also be linear when the coordinates are logarithmic Hence, for a given urea concentration extrapolation is possible to the inhibition at substrate concentrations too low to be measured

2 *Irreversible Inhibitors*

Paroxan[1]) (0 0-diethyl-0 p nitrophenyl phosphate) was chosen as irreversible inhibitor Under magnetic stirring 25% human haemolysate was incubated for 3 hours at 38° and neutral reaction with 1/300 µg paroxan per millilitre (cf JENSEN-HOLM 1960) After cooling to 0°, haemolysate was taken for analysis (1 ml diluted to 25 ml) Compared with the corresponding normal values, *the degree of inhibition obtained was nearly the same, no matter which substrate contration had been used* (from 10^{-5} M to 4×10^{-3} M ACh), namely, about 50% of the normal activity

Discussion.

A *Method*

It has been shown in the work discussed here that introduction of a Twin Assembly for specially fixing two identical and simultaneously acting syringes makes it possible to operate at even low substrate concentrations The hydrolysis of acetylcholine is seen graphically as a process of zero order at high concentrations, because the loss of acetylcholine is negligible At low concentrations on the other hand, the loss is of significance, and the process becomes graphically one of the first order The introduction of the twin syringe burette, one syringe containing the titration fluid (base) and the other acetylcholine at equimolar concentrations, renders possible fairly exact measurements and the curves will appear as if the process were of zero order

We have recently found that HEILBRONN (1958) has made use of an apparatus resembling in principle that described above for analyses of purified enzyme preparations However, with blood and tissue homogenates employment of the twin syringe burette presupposes that, before and during the analysis, it is possible to control the often pronounced spontaneous, unspecific acid or base production, which otherwise may render analytic measurements of [ChE] impossible In our study we used a constantly working infusion apparatus with an adjustable rate of ad-

[1]) The paroxan used which is chemically pure (about 99%) was kindly placed at our disposal by *Farbenfabriken Bayer, Leverkusen* Germany through Professor W WIRTH

vance of the piston (lowest rate corresponds to about 3 μ/sec and highest to about 40 μ/sec), to obtain an artificial small excess of acid, manifesting itself as a slight blank activity on the curve. The sensitivity of measuring [ChE] depends on the possibility of keeping the blank activity both constant and low in proportion to the total activity.

B. *Normal Cholinesterase Activity*

Figs. 2, 3, and 4 show the cholinesterase activities in three normal tissues. It is true of all these that, when the substrate concentration falls, the activity decreases steadily, approaching asymptotically to zero. At substrate concentrations below 10^{-4} M, [ChE], plotted logarithmically against pS will for all three tissues give a straight line, which renders extrapolation possible. The justification for this is further seen in fig. 9, where both axes represent arithmetical values. The curve is seen to tend towards zero.

Plotting the results, obtained by HOFSTEE's (1952) variation of MICHAELIS & MENTEN's (1913) equation, for [ChE] (ordinate) against [ChE] divided by C_S (abscissa), it has been shown that straight lines should be obtained (cf. AUGUSTINSSON 1948, HEILBRONN 1958). However, such linear courses were seen nowhere by us. Likewise $\frac{1}{[ChE]}$ plotted against $\frac{1}{C_S}$ should give straight lines but did not do so. On the other hand, $\log \frac{1}{[ChE]}$ plotted against $\log \frac{1}{S}$ was seen to have a linear course at substrate concentrations below about 10^{-4} M. This is the same as saying that log [ChE] plotted against pS forms a straight line, because $\log \frac{1}{[ChE]} = -\log [ChE]$ and $\log \frac{1}{C_S} = pS$. Thus, the enzyme activity does not follow the equation

$$[ChE] = -\frac{[ChE]}{C_S} K_M + [ChE]$$ (vide AUGUSTINSSON 1948, among others).

In addition the experimental investigations have shown for erythrocyte cholinesterase that at substrate concentrations above 10^{-3} M [AChE] plotted as ordinate against $\log \frac{[AChE]}{C_S}$, gives a straight line. This has been interpreted as "inhibition by excess of substrate", an inhibition that perhaps already begins to manifest itself close to the first point of inflec-

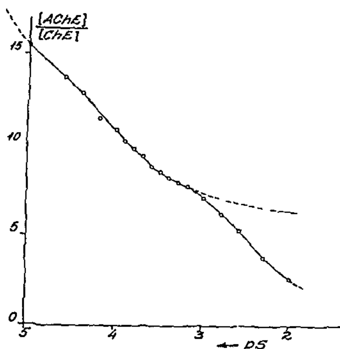


Fig 8 [AChE] and [ChE]
The chart shows the ratio between [AChE] from erythrocytes and [ChE] from plasma (same human subject Values from figs 2 and 3) related to rising substrate concentration
Ordinate $\frac{[AChE]}{[ChE]}$
Abscissa negative logarithm of substrate concentration (= pS)
The broken line indicates the assumed course if there had been no inhibition by excess of substrate of [AChE]

tion (pK_1), where the substrate concentration is just over 10^{-4} M The change taking place here must be due to at least two factors

a) auto-inhibition of substrate,
b) shortest time for the individual stages of the whole process and the chance of contact with a new acetylcholine molecule

Both factors operate for the specific cholinisterase, whereas for the unspecific enzyme only the latter seems to do so We have tried to demonstrate this by plotting the erythrocyte esterase activity devided by the plasma esterase activity (ordinate) against the substrate concentration (logarithmically ∾ pS), see fig 8 The figure shows that the lower the substrate *concentration, the relatively more effective is the erythrocyte* cholinesterase activity *The* same is seen in fig s *2* and *3*, where we find from the logarithmic course that [AChE] decreases 5 1 times and [ChE] 6 9 times with each ten fold reduction in substrate concentration *How-*ever, in relation to the substrate concentration, both activities are relatively increased

Fig 8 demonstrates further that the curve bends between pS = *3*

and = 4. If no auto inhibition existed, the course would probably have been as indicated by the broken line. The unbroken curve, which represents the course in fact taken, shows, compared with the broken one, an inhibition that becomes more and more pronounced the higher the substrate concentration. This way of illustrating substrate inhibition is certainly somewhat unorthodox and hardly above criticism, but the result seems convincing. In relation to the present study it must, however, be regarded as of purely academic interest.

C. [ChE] in the Presence of Inhibitors

It is clear from our results that alterations in substrate concentration cause no relative changes in cholinesterase activity *when irreversible inhibitors are used. The degree of inhibition obtained is nearly the same throughout, irrespective of the substrate concentration chosen and irrespective of the dilution of any tissue incubated in advance.*

With *reversible inhibitors*, on the other hand, the strength of the substrate concentration may fundamentally affect the degree of inhibition obtained. The results given in fig. 5 show that at pS = 3.4 the inhibition is nearly the same for all three inhibitors used. The sensitivity to alterations in substrate concentration is greatest for the inhibitors with the lowest specificity (in this instance, ether). With physostigmine the changes are certainly far less pronounced, though still significant. It is seen from fig. 5 that the measured activity as a percentage of the corresponding control activity is four times greater at pS = 2.40 than at pS = 5.27 (30 and 7.5%, respectively of the control values).

The same applies to urea as to ether, except that urea must be regarded as an even weaker inhibitor than ether. Some of the results are given in figs. 4, 7, and 9. The degree of inhibition obtained depends directly on the substrate concentration. At a fixed concentration, the inhibitions due to urea, ether or morphine expressed logarithmically and plotted against pS give straight lines to the left of the points of inflection of the curves. This renders extrapolation possible. The same may also be true of physostigmine.

We have compared the results achieved by TORDA (1943) with those recorded above for the experiments with ether as cholinesterase inhibitor. She worked with an acetylcholine bromide concentration of about 1.3×10^{-2} M and used a modification of the method developed by GLICK (1938). At ether concentrations of 1.5 and 3.7% (v/v) she found a residual activity of 82 and 75%, respectively, of the corresponding control value, in other words, there was only a slight reduction in enzyme activity. At the substrate concentration mentioned, the results agree, despite the difference in method, with those got by extrapolation of the

curve in fig 5 At the same time the results show that conclusions about conditions and activity *in vivo* may be drawn only with care from the *in vitro* experiments

As is evident from the results, morphine is a cholinesterase inhibitor manifesting increasing inhibitory action the lower the substrate concentration It is difficult to decide whether or not the acute toxicity of morphine is due in part to cholinesterase inhibition In this connection there may be some relevance of an investigation by BASHFORD (1901), who in experiments on albino rats showed plainly that atropine in suitable doses (few mg/kg) can raise the lethal dose of morphine (M L D), whereas larger doses have no such effect

It is impossible to predict whether a reversible inhibitor will possess pharmacological and toxicological functions as a cholinesterase inhibitor *in vivo*, if the cholinesterase activity is only determined at a high substrate concentration It may be added that any dilution of tissue or blood for the reaction mixture involves a corresponding reduction in inhibitor concentration The inhibition depend closely on the inhibitor concentration A decrease of this means a shift to the left of the equilibrium $E + I \rightleftarrows EI$ (E = enzyme and I = inhibitor) As it is necessary for the determination of [ChE] to operate at low tissue concentrations we must therefore proceed by first determining the concentration of the inhibitor in the drawn undiluted tissue specimen and then adding inhibitor to the reaction mixture in such amounts that the same concentration is obtained in the reaction mixture as in the undiluted tissue

In studies on chlorpromazine (JOHANNESSON & LAUSEN 1961) and morphine (JÖHANNESSON 1962) account was taken of the above facts by first determining the inhibitor concentration in the tissue specimen taken and then working with the same concentration in the reaction mixture Further, these workers varied the substrate concentration to achieve a more exact evaluation of the actions of these drugs as cholinesterase inhibitors

To determine pharmacologically the action of an inhibitor it is important to know *the substrate concentration existing in vivo* physiologically or pathologically changed e g by toxic agents These concentrations are unknown, however, and we have had no opportunity so far to measure them

According to ACHESON's (1948) survey of measurements of liberated acetylcholine per end plate or synapse in cat and frog, this is about 1.5×10^{-10} µg ACh (base) per impulse corresponding to 600,000 molecules WASER (1960) is of the opinion that a certain loss must take place He therefore regards 10^6 molecules as a more correct value If we take the nerve surface of the end plate to be 500 µ² (WASER 1948) and the

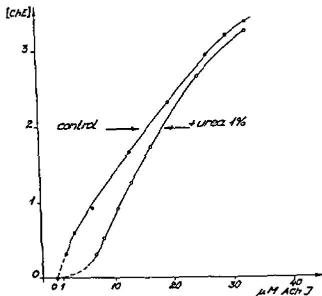


Fig 9 Brain from guinea pig Same experiment as illustrated in fig 4

Ordinate [ChE] in μmol/min /g brain tissue

Abscissa The micromolar (μM) concentration of acetylcholine iodide expressed arithmetically

Lower curve same experiment in presence of urea at a concentration of 1% (w/v)

The figure shows that the normal curve tends towards zero, a fact supporting the postulate about extrapolation (for details see text)

distance to the teloglia about 5 μ, we deduce the presence of an open space of 2500 μ^3 at most The concentration of acetylcholine liberated by a single impulse, supposed to be evenly distributed in this space, must thus be not less than 6.6×10^{-7} M, or nearly 10^{-6} *M*

STEWART (1952) has measured the concentration of ACh in brain tissue from animals killed with TEPP (tetraethyl pyrophosphate) and animals killed by asphyxia He found it to be 4 35 μg/g and 2 69 μg/g, respectively This amount, however, according to STEWART includes bound (precursor) as well as liberated acetylcholine From the former value the molar concentration on the assumption of even distribution, has been calculated to be 3×10^{-5} *M*

In the blood of rats poisoned with paroxan (= paraoxon), STEWART (1952) found between 8 and 30 ng ACh (base) per millilitre Others (DOUGLAS & PATON 1951) have found about 40 ng/ml in cats poisoned with TEPP BARNES & DUFF (1954) in paraoxon poisoned animals, [illegible] 20 ng/ml (dogs), about [illegible] words, the values were [illegible] about 2×10^{-7} *M*

Thus, there are three values available The last one (2×10^{-7} M) must be regarded as the *lower limit*, and the total concentration in brain tissue (3×10^{-5} M) presumably as the *upper limit* The first value (about 10^{-6} M) is the probable concentration round the end-plate, or synapse, though we cannot say to what extent adsorption may influence the physiological concentration

For the present we must therefore aim at establishing the enzyme activity as closely as possible to 10^{-6} M ACh This is important, first, because the rate of hydrolysis decreases with falling substrate concentration (at 10^{-6} M [AChE] constitutes only 1 8 per cent of the highest activity), and, secondly, because in the presence of reversible inhibitors we obtain a degree of inhibition that can be far more closely related to different pharmacological maximal Functions If the physiological concentrations ultimately turn out to be, for instance, lower than we have here supposed, e g 10^{-7} M, in other words, outside our range of measurements, we shall still have a fair chance of extrapolating by means of analyses to the cholinesterase activity, both in absence and in presence of inhibitors This can be done by employing the logarithmic, linear course demonstrated in our work for low substrate concentrations

For normal values such extrapolation really does seem to give correct values, as we have tried to illustrate in fig. 9, where both axes are arithmetical the line running a curved course tends distinctly towards zero

Summary.

1 A method is described for exact measurement of cholinesterase activity at substrate concentrations down to 10^{-6} M The substrate concentration in the reaction mixture is kept constant by means of a Twin Assembly attached to the titrigraph and carrying two identical glass syringes containing titration fluid (NaOH) and acetylcholine iodide in equimolar concentrations Measurements performed on homogenates of blood and tissue presuppose thorough control of spontaneous, non specific acid/base production by means of a special infusion apparatus

2 The results of studies on cholinesterase activity in normal tissue are shown At substrate concentrations below about 10^{-4} M the activity curve, plotted against the substrate concentration, is linear when both axes are set out logarithmically The curves have been shown not to follow the Michaelis-Menten's equation

3 The degree of inhibition after incubation with irreversible inhibitors is independent of the substrate concentration employed as well as of subsequent tissue dilution

4 The degree of inhibition after incubation with reversible inhibitors depends on the substrate concentration and the sensitivity to alterations in substrate concentration is greater the less specific the inhibitor used

5 At substrate concentrations lower than the points of inflection in the activity curves in the presence of inhibitors the curves have linear course when the residual activity (as percentage of the control value) as well as the substrate concentration are expressed logarithmically This renders extrapolation possible

6 The physiological concentration of acetylcholine is estimated at about 10^{-6} M

7 In studying cholinesterase activity in absence or presence of inhibitors one should work at low substrate concentrations so as to be able more correctly to evaluate the physiological enzyme activity and to form a better quantitative evaluation of the pharmacological action of cholinesterase inhibitors *in vivo*

Acknowledgement

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Chlorpromazine as an Inhibitor of Brain Cholinesterases

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A side-effect of treatment with chlorpromazine (and many other phenothiazine derivatives) is the development of *excitation*, which, particularly in epileptoid individuals, may result in convulsions (FAZEKAS *et al* 1957) In adults this side-effect is generally seen only after administering larger doses (400–800 mg daily), but children seem to be considerably more sensitive (SHAW 1960) The extrapyramidal signs and symptoms predominate in adults (WILSON & WOOD 1955) A considerable number of patients develop an unmistakable PARKINSON'S SYNDROME (MOYER *et al* 1955, COHEN 1955), however, this usually subsides within a few days when treatment is discontinued As a rule chlorpromazine and other phenothiazine derivatives have a pronounced sedative action, being used in the treatment of those conditions, e g Parkinson's disease, in which morbid restlessness and agitation are the most conspicuous features Chlorpromazine has therefore been characterised as a drug with a paradoxical action (cf WUTSCHKE 1960) *Similar paradoxical actions* are known for various other drugs (e g antihistamines, barbituric acid derivatives and reserpine)

Chlorpromazine is known to act on numerous enzymic systems, e g dehydrogenases and cholinesterases The inhibition of serum cholinesterases seems the action to have been most thoroughly investigated (cf VIAUD 1954) GORDON (1948) found that diethazine a phenothiazine derivative, was an inhibitor of cholinesterase activity in plasma Several workers (FOURNEL 1950, COURVOISIER *et al* 1952, MORAND & GAY 1953, TODRICK 1954, LIŠKA 1957, LINDAUR & ZELENY 1958, HOFSTEE 1960, EVANS 1960) have since confirmed this observation for other phenothiazine derivatives, among which is chlorpromazine On the other hand, GORDON noticed that *diethazine did not inhibit cholinesterases in rat brains* He was supported by TODRICK, who claimed that ethopropazine

and promethazine as well as diethazine seem to be specific inhibitors of cholinesterases in the intestinal mucosa, i e inhibitors of the so-called pseudocholinesterases However, these views are inconsistent with the results of our experiments with chlorpromazine, described below

WUTSCHKE (1960), surveying the toxicology of chlorpromazine, points out that it and other phenothiazine derivatives seem to be more toxic to children than to adults Thus, DONE (1960) has described a case of severe, though not fatal, poisoning of a 4 year old boy after ingesting 80 mg, at most, of a phenothiazine derivative (prochlorperazine), and FERGUSON (1957) and ANDREASSEN (1959) have reported cases of two adults who attempted suicide by taking about 4 5 g and 5 g chlorpromazine and survived In DONE's case there were marked extrapyramidal signs and symptoms, but in the one described below the condition was more like that characteristic of poisoning due to potent cholinesterase inhibitors, such as organic phosphorus compounds

Because of a case of *fatal chlorpromazine poisoning* in infancy examined by us, we studied the ability of chlorpromazine to inhibit cholinesterase activity in brains The results are discussed and considered in relation to the so-called paradoxical action of this drug

1. Case Report.

Serial Number 2251 A girl, aged 11 months, consumed an unknown amount of chlorpromazine (largactil ® tablets) Nearly 12 hours later fasciculations occurred in her eyelids and the muscles of the extremities (in the case record of the hospital described as twitchings of all extremities and eylids) The fasciculations continued despite treatment with barbituric acid derivatives and later with atropine and a calcium preparation At the end she was deeply unconscious and cyanotic she still had fasciculations of the eyelids and hand muscles, and death occurred 19 to 20 hours after intake of the poison

at [illegible]

ar [illegible]

potent cholinesterase inhibitors, such as e g physostigmine, nitrostigmine (– parathion) and other "phosphostigmines" For this reason we determined the cholinesterase activity in whole blood from the dead infant using a modification of MICHEL's electrometric method (MICHEL 1949) The measured pH change per hour was 0 95 This value is within the range found for normal blood in this Department by the same method (0 7–1 1)

Gastric contents, liver tissue and brain tissue were analysed for chlorpromazine by the method of SALZMAN & BRODIE (1956) In calculating the concentrations in the tissue specimens, allowance was made for the fact that these authors, after additions *in vitro*, recovered about 90% of the added amount from most tissues, but only about 40% from brain

Employing corrections we found
about 210 µg chlorpromazine chloride per millilitre of gastric contents,
about 135 µg per gram liver tissue, and
about 200 µg per gram brain tissue

There was no evidence to suggest presence of the conversion product chlorpromazine-sulphoxide

2. Methods.

The procedure employed for cholinesterase determination is based on that of JENSEN-HOLM, LAUSEN, MILTHERS & MOLLER (1959) However, various modifications of an instrumental nature have made it possible to obtain reliable results with substrate concentrations several hundred times lower than those usually employed

A *Apparatus*

The activity was measured by continuous, automatic titration with titrator TTT1a and titrigraph SBR2a/SBU1 both manufactured by RADIOMETER The titrigraph is equipped with Twin Assembly DS 60083 carrying two simultaneously working Agla syringe burettes instead of one (developed by JENSEN-HOLM in collaboration with RADIOMETER) By means of an infusion apparatus independent of the titrator, other reagents can be added to the test mixture at a rate that can be kept constant for a considerable time (JENSEN-HOLM 1961)

B *Reagents*

The titrant used was 5 mM NaOH whose strength was controlled by titration of potassium hydrogen tartrate One Agla syringe contains the sodium hydroxide and the other an equimolar solution of the substrate i e 5 mM of acetylcholine iodide The enzymatic process is started by adding from a pipette a suitable volume of this solution to the test mixture The chlorpromazine is added as a 100 mM aqueous solution of the chloride Air free redistilled water is used for the analysis

C *Materials*

Rat brain Two male albino rats (about *180 g*) were decapitated and their brains (incl the brain stem and the cerebellum) examined jointly

[illegible]

at −20 C during the interval between autopsy and preparation On the latter occasion, tissue specimens were taken from

a) the cerebellum,
b) the deep layers of the cortex and the basal ganglia,
c) the cortex of the temporal lobe

The tissue specimens chosen were examined separately

The specimens were *homogenised* mechanically for 1 or 2 minutes with a suitable volume of 0 9% NaCl solution The temperature was kept low before, during and after homogenisation by means of an ice bath The homogenate was diluted with 0 9% NaCl solution to a suitable volume, i e to a homogenate concentration of 25–30% w/v

3. Estimations of Activity.

For the activity estimations we used 0 5 ml of rat brain homogenate or 2 0 ml of infant brain homogenate diluted with sufficient 0 9% NaCl solution to bring the reaction mixture (after addition of inhibitor and substrate) to a total volume of 100 ml Nitrogen was bubbled through the mixture kept at 38°C, and while estimating the activity, during determination of the activity with the reaction vessel kept covered, we swept out the air space above the reaction mixture with nitrogen to avoid absorption of carbon dioxide The activity was determined at pH 7 40 with constant magnetic stirring

The amounts of substrate and chlorpromazine in the aggregate reaction mixture are shown in table 1

After adjustment of the test mixture to pH 7 40 for the purpose of determining cholinesterase activity in tissue specimens, a non-specific liberation of acid (falling pH) or of base (rising pH) is often seen before the addition of substrate In such events the enzyme activity recorded after addition of substrate will be too high or too low, respectively. To avoid these sources of error a weak solution of sodium hydroxide or of hydrochloric acid is added continuously by means of the infusion apparatus mentioned above While working on these problems we found that activities (or, more correctly, titrant addition rates) below a certain value were not recorded satisfactorily (possibly because of the apparatus), whereas activity *differences* of the same order could be measured with great [illegible] adjusted so as to [illegible]", the size of which [illegible] id, and non-specific acid, or base, liberation, respectively Then, after adding the substrate, the enzymatic activity will be recorded as well The use of this technique presupposes that addition of substrate does not influence the "blank activity" by altering the non specific acid or base liberation

However, the experience gained so far suggests that no such alterations take place (vide JENSEN-HOLM, LAUSEN, MILTHERS & MOLLER 1959) It has, moreover, been found that the technique described above makes possible the recording of extremely low enzyme activities with considerable accuracy (cf fig 1)

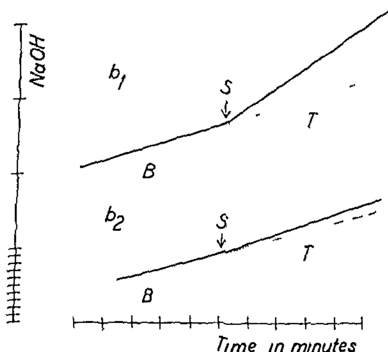


Fig 1 Two examples of titration curves
The curves refer to b_1 and b_2 in table 1 B and T denote blankactivity and total activity respectively
S indicates the addition of substrate (acetylcholine) The curve for blank activity is continued as a dotted line, thus illustrating the increase in activity after addition of substrate
Abscissa time in minutes
Ordinate sodium hydroxide added One small unit corresponds to 0 026 µmole NaOH

By means of the twin syringe burette, equivalent amounts of base and substrate are added simultaneously *This makes it possible to maintain a constant substrate concentration in the reaction mixture*, a fact of particular importance at low substrate concentrations, when high enzyme activity would otherwise quickly split an appreciable amount of the substrate present During pre-titration to pH 7 40, the simultaneous addition of substrate is suspended

For practical reasons the brain tissue was incubated with chlorpromazine for 10 to 20 minutes before substrate was added, even though pilot experiments had shown that the equilibria of enzyme inhibitor substrate adjust themselves almost immediately

The enzyme activity is expressed as micro moles of liberated acid per minute per gram tissue ($\mu mol \times min^{-1} \times g^{-1}$)

4. Results.

Rat brain. With a substrate concentration of 1.0×10^{-5} M the activities found after incubation with chlorpromazine for 10 minutes were

without chlorpromazine	0.80 μmol/min./g
1.1×10^{-4} M chlorpromazine	0.44 μmol/min./g
1.0×10^{-5} M chlorpromazine	0.85 μmol/min./g

Thus, a concentration of 1.1×10^{-4} M, or about 39 μg chlorpromazine chloride per millilitre, inhibited the enzyme by nearly 50 per cent, whereas a concentration of 1.0×10^{-5} M (or 3.6 μg/ml) did not inhibit cholinesterase activity in rat brains.

Infant brain. Larger amounts of brain tissue were found to be required here than in the rat brain experiments to obtain a suitably high activity. The substrate concentration was in all experiments 1.25×10^{-5} M. In those with chlorpromazine the brain tissue was incubated with it for 15 minutes before addition of substrate. The concentrations used and the activities measured are listed in table 1.

Table 1

Effect of Chlorpromazine on Cholinesterase Activity in Homogenates of Human Brain (child)

a tissue from cerebellum
b tissue from the deeper layers of the cortex and from the basal ganglia
c tissue from the cortex of the lobus temporalis
Substrate: 1.25×10^{-5} M acetylcholine iodide
Activity in μmoles per minute per gram tissue

Tissue	Chlorpromazine in molarity	Cholinesterase activity	Remaining activity as percentage
a_0		0.33	100
a_1	1×10^{-4}	0.13	39.5
a_2	3×10^{-4}	0.021	6.4
b_0		0.14	100
b_1	1×10^{-4}	0.062	44.2
b_2	3×10^{-4}	0.01	6.8
c_0		0.16	100
c_1	1×10^{-4}	0.075	47.0
c	5×10^{-4}	not measurable	

5. Discussion.

When analysing cases of poisoning in which symptoms of impaired cholinesterase activity are observed, we must distinguish sharply between reversible and irreversible inhibitors Such a distinction has been made in all enzymatic studies on these substances, whereas the pharmacological and toxicological consequences of this have remained surprisingly unheeded This problem is further discussed below

Inhibition of cholinesterases by *irreversible inhibitors* of the phosphostigmine group is a reaction resulting in production of stable enzyme-inhibitor compounds, which can only be split in special circumstances

The results of inhibition by *reversible inhibitors*, e g physostigmine and morphine, are entirely different a reversible equilibrium is established between enzyme and inhibitor *Any dilution of the system, as also addition of other inhibitors or of a substrate, will cause dissociation of the original enzyme-inhibitor compound to a greater or lesser degree* All current methods of determining cholinesterase activity involve dilution and addition of substrate to a considerable extent Consequently the activity measured is certainly higher than that of the tissue *in situ*

KUHN & SURLES (1938) investigated the inhibiting action of morphine (among other drugs) on brain cholinesterases They showed that at a constant concentration of morphine the inhibition increased with decreasing substrate concentration and *vice versa* Experiments conducted in this Department have borne out the reported observations with morphine (JÓHANNESSON 1962), and our investigations have shown that numerous other drugs may effect reversible inhibition of cholinesterases in different tissues (JENSEN-HOLM 1960 1961)

After administering large doses of morphine JÓHANNESSON determined the cholinesterase activity in rat brains as described below On the basis of a chemical determination of morphine in specimens of the brain tissue whose cholinesterase activity was to be determined, enough morphine was added to the reaction mixture to ensure that the consequences of the necessary dilution of the tissue were eliminated By using as low substrate concentrations as 10^{-6} to 10^{-5} M the dissociation due to the addition of substrate could be almost avoided

Chlorpromazine gave analogous reactions in rat brain and infant brain (table 1) At a concentration of 3×10^{-4} M *chlorpromazine, about 90* per cent inhibition was found, at a concentration of 5×10^{-4} M, the activity was so low as to be unmeasurable That GORDON (1948) found no inhibition in response to the phenothiazine derivative tested by him at a concentration of 5.1×10^{-4} M was probably due to the fact that he worked with acetylcholine at a concentration of 7×10^{-3} M, i e a concentration nearly a thousand times higher than that used by us

We might therefore expect *a priori* that in the case reported above whole blood would show a normal cholinesterase activity by the modification of MICHEL's method described By this procedure the blood is diluted about 60 times and the substrate concentration is high, about 1.6×10^{-2} M acetylcholine It may be added that the blood concentration of chlorpromazine in the organism falls rapidly to a low level (SALZMAN & BRODIE 1956) In fact normal cholinesterase activity was found This finding precluded apparently poisoning by phosphostigmines, of which the clinical signs had otherwise been suggestive

The forensic-chemical analysis revealed the presence of chlorpromazine in the brain tissue The concentration was calculated as about 200 µg per gram brain tissue The corresponding molar concentration is about 6×10^{-4} M, a concentration that, according to our results, completely checks cholinesterase activity in the brain

It is accordingly reasonable to suppose that the clinical signs observed in the poisoned infant were in fact of cholinergic origin

In this connection mention should be made of some experiments by VLK (1957) After administering large doses of chlorpromazine to rats (about 20 mg/kg intraperitoneally), he noted that more acetylcholine could be extracted from the atria of their hearts than from those of controls Subsequently VLK (1958) also found that chlorpromazine had no effect on cholinacetylase activity The rise in acetylcholine concentration of the atria is therefore more probably explained by a reduced hydrolysis of the acetylcholine than by an increased synthesis

JENKER & WARD (1953) incline to the view that the signs and symptoms in Parkinson's disease are of cholinergic origin, RINALDI & HIMWICH (1955 a, b) have shown that the reticular apparatus of the brain acts cholinergically They conclude (1955 c) that small doses of chlorpromazine inhibit the reticular apparatus, whereas large doses stimulate it

Hence, there is reason to suppose that the "paradoxical" action of chlorpromazine is due to the cholinesterase inhibition caused by the high concentrations and the consequent stimulation of the reticular apparatus of the brain

Summary.

1 In an infant dead of fatal poisoning, the signs and symptoms of which suggested a cholinergic origin, high concentrations of chlor-

[illegible] as found to be a potent reversible inhibitor

of brain cholinesterases. The chlorpromazine concentration of about 200 µg/g (about 6×10^{-4} moles per kilogram tissue) found was seen *in vitro* completely to inhibit the cholinesterase activity in brain tissue from an infant.

3. It is concluded that the excitation seen as a side-effect of chlorpromazine treatment (the "paradoxical" action) can be interpreted as of cholinergic origin.

4. *The pharmacological and toxicological importance of distinguishing* sharply between reversible and irreversible cholinesterase inhibitors is stressed. When determining cholinesterase activity after inhibition by reversible inhibitors, it is characteristic that dilution as well as addition of substrate reduces or eliminates the inhibition. Thus the finding of normal cholinesterase activity *in vitro* by no means precludes the observed signs and symptoms being due to impaired cholinesterase activity *in vivo.*

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From the Department of Pharmacology (Professor Gunnar Ahlgren, M D)
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Fluorescence of D,L-Aldosterone in an Acid Medium

By

Paio Hedner

(Received November 13 1961)

The fluorescence of certain steroids in strong acid is well known and has been used in methods for quantitatively determining e g corticosterone, cortisol (hydrocortisone) (SILBER *et al* 1958, GUILLEMIN *et al* 1958) and some oestrogens (BATES & COHEN 1950, DICZFALUSY 1953) However, no appreciable fluorescence occurs on dissolving other steroids, e g cortisone or aldosterone, in acid medium The reasons for this difference are obscure It is not known with certainty which properties of the steroid molecule are responsible for developing fluorescence The intensity and possibly other properties of the fluorescence may vary and depend on the kind of acid used, its concentration and other factors For instance, the fluorescence of oestrone and oestradiol is intensified by heating the acid solutions (BATES & COHEN 1950, DICZFALUSY 1953) It is also known that the properties of aldosterone in sulphuric acid solution are changed after heating Thus SIMPSON *et al* (1954) demonstrated one absorption peak of aldosterone dissolved in concentrated sulphuric acid at 288 mμ, whereas after heating the same solution to 90 C for one hour peaks were detected at 247, 285, 342 and 460 mμ

Because of these findings it was thought of interest to establish whether or not fluorescence of D L-aldosterone in acid solution could be produced by heating the solution

Material and Method.

An aqueous solution of D L-aldosterone*, 0 5 mg per ml was used This was diluted with absolute alcohol to a concentration of 10 μg per ml and 1 ml of this was evaporated under reduced pressure to give dry samples of 10μg D L-aldosterone

Synthetic cortisol was dissolved in absolute alcohol at a concentration of 0 2 µg per ml and evaporated to give dry samples of 0 2 µg

Concentrated sulphuric acid, p a , diluted with absolute alcohol in the proportion 75/25 (v/v) was used as acid medium throughout the experiments

Fluorescence measurements were carried out with an Aminco-Bowman spectrophotofluorometer at an activating wavelength of 465 mµ and a fluorescent wave length of 550 mµ

The evaporated aldosterone samples were dissolved directly, usually in 4 ml of sulphuric acid/ethanol medium, if other concentrations were needed, further dilutions with the acid medium were made before heating

Of the acid solution 2 ml in a glass tube were placed in a hot water bath The remaining 2 ml were poured into a cuvette After a suitable time the heated test tubes were removed from the bath and cooled to 18°C in another water bath After cooling the solutions were examined in the spectrophotofluorometer alongside the unheated parts of the samples A 0 1 µg solution of cortisol in acid medium was used as a reference standard in some experiments for calibrations of the apparatus, as its fluorescence can be considered as constant for at least 20 minutes (HEDNER 1961)

Results.

With the H_2SO_4/ethanol mixture as the solvent, it was soon evident that some fluorescence was developed by D,L-aldosterone after heating a solution that had previously shown little or no fluorescence The fluorescence maximum of the heated solution was situated at 465/550 mµ Cortisol showed the same maximum, and the picture of the fluorescence pattern of aldosterone on the cathode ray oscillograph was closely similar to that of cortisol

Optimal Time of Heating

To find the most suitable time of heating, a number of aldosterone solutions at three concentrations, 5, 1 and 0 2 µg per ml, were placed in the water bath adjusted to 70°, removed, cooled and measured at different time intervals An unheated solution of cortisol was used as a reference standard, its scale reading being adjusted to 80

The results are shown in fig 1 The values for the unheated solution did not differ much from each other or from the sulphuric acid/ethanol blank value After heating for 5 or 10 minutes, the readings were considerably higher They also showed differences between the three concentration levels, the ratios of the readings corresponding well with those of the concentrations The sulphuric acid/ethanol blank showed slightly higher readings after heating

The fluorescence of the cooled aldosterone solutions proved to be stable for at least 20 minutes

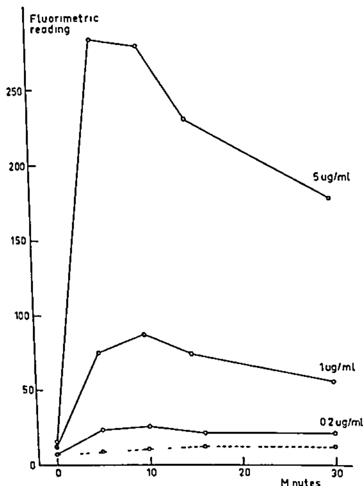


Fig 1 Influence of heating time on the development of fluorescence of D L aldosterone in sulphuric acid/ethanol medium at 75°C Broken line represents the sulphuric acid/ethanol blank

Unheated aldosterone solutions revealed no fluorescence after 10, 20 or 30 minutes

After 15 minutes heating, the fluorescence of the aldosterone solutions showed a tendency to decrease, as did the readings after 20 and 30 minutes

The optimal time for heating under these conditions was thus held to be 5–10 minutes In the experiments described below the heating time was 5 minutes

Optimal Temperature

A number of aldosterone samples, dissolved in the acid medium at a concentration of 1 0 µg per ml, were heated for 5 minutes to different

Synthetic cortisol was dissolved in absolute alcohol at a concentration of 0 2 µg per ml and evaporated to give dry samples of 0 2 µg

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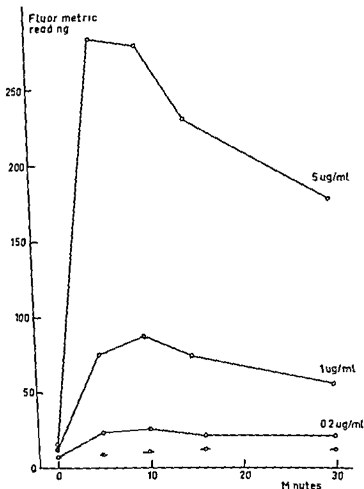
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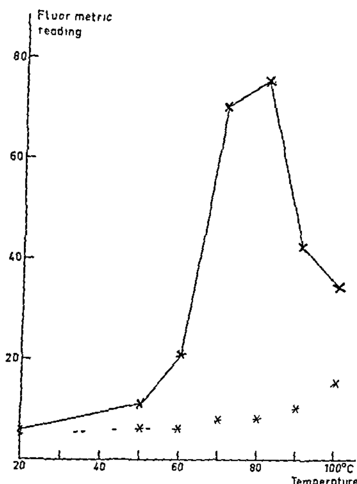


Fig 2 Influence of temperature on the development of fluorescence of D L aldosterone in sulphuric acid/ethanol medium Heating time 5 minutes Broken line represents the sulphuric acid/ethanol blank

temperatures After cooling the samples were examined in the spectro photofluorometer together with the cortisol standard The results are shown in fig 2 A suitable temperature is evidently 75°, and this was used in the further experiments

Concentration Fluorescence Curve

It was possible to obtain a straight line as plotting concentration against fluorescence in the range 0 2–1 0 µg per ml (fig 3) As at least 2 ml are required for a determination, the smallest aldosterone sample possible that can be measured by this method is 0 4 µg

Specificity Remarks

To obtain more information about the specificity of the aldosterone fluorescence reaction to heat, several common steroids were investigated

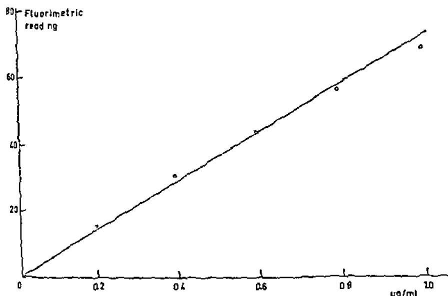


Fig 3 Concentration fluorescence curve for D L aldosterone in sulphuric acid/ethanol medium heated to 75°C for 5 minutes

Table 1

Fluorimetric scale readings before and after heating (75 C, 5 minutes) several steroids dissolved in sulphuric acid/ethanol medium The values are corrected for blank readings

Substance	Concentration µg per ml acid medium	Fluorimetric reading *before* heating	Fluorimetric reading after heating	Fluorescence maximum Activating/fluoresce wavelength, mµ
Cortisol (Hydrocortisone)	0 1	76	27	465/550
Corticosterone	0 1	165	20	465/550
D L Aldosterone	5 0	7	280	465/550
Deoxycorticosterone acetate	5 0	28	137	465/545
Oestrone	0 2	18	about 500	450 500
Oestriol	2 0	35	420	450 500
Oestradiol	0 2	78	390	450/500
Testosterone	5 0	8	16	
Cortisone	5 0	4	1	
Prednisone	5 0	26	41	465/555
Prednisolone	5 0	0	1	
Dexamethasone	5 0	1	5	

The results are shown in table 1, from which it can be seen, that most of them showed minute or no increases in fluorescence intensity The fluorescence of hydrocortisone and corticosterone decreased considerably

Like the fluorescence of aldosterone, that of desoxycorticosterone acetate increased on heating, but to a less extent (from 27 scale units before to 137 after) The peaks for the fluorescence maxima seemed not to change after heating for any of the substances investigated

Discussion.

As pointed out above, the concentration fluorescence curve for aldosterone is linear over an appreciable range This makes it possible to adapt the heat-induced fluorescence reaction to quantitative aldosterone measurements, especially as this fluorescence proved to be quite stable The sensitivity offers, however, scarcely any advantages compared with, e g, the measurement of aldosterone fluorescence in alkaline medium, which permits the detection of aldosterone in about the same quantities (STAUB *et al* 1961) However, it should adequate for determining aldosterone in urine The specificity of heat induced aldosterone fluorescence is not absolute, as can be seen from table 1 Deoxycorticosterone acetate gave a similar reaction, and there are perhaps other substances that act in the same way The employment of aldosterone fluorescence in acid medium as the basis of a quantitative method thus necessitates suitably purified aldosterone extracts or at least extracts containing only known contaminants If these criteria are fulfilled the method can perhaps offer some simplification in urinary aldosterone determination The purification of the urinary extract, after passage through a florisil column, usually includes several paper chromatographic stages The last of these often involves the separation of aldosterone and cortisone (see e g LABADIE 1959)

In the acid fluorescence method this step could possibly be omitted However, there are in this fraction besides aldosterone and cortisone, small amounts of other unknown substances sometimes called "the amorphous fraction", whose fluorescence is not yet known

Summary.

The development of fluorescence by D,L aldosterone in acid medium after heating is described The optimal time and temperature are given A linear concentration fluorescence curve was obtained

The conditions and possibilities for adapting the method to quantitative aldosterone determinations are discussed

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The Separation of Alkaloids from Extracts of Animal Tissues; the Use of Cation Exchange Resins.

By

S. L. Tompsett

(Received August 28 1961)

The results of an investigation into the separation of alkaloids from urine by the use of a cation exchange resin (Dowex 50 × 12) have been reported in a previous paper (TOMPSETT 1960 a) The present paper describes an extension of these studies Separation of alkaloids from tissue extracts has been examined and also the elution of alkaloids bound to the cation exchange resin

General Methods.

These are similar to those reported in the previous paper

The Preparation of Cation Exchange Columns

The cation exchange resin Dowex 50W × 12 (200–400 mesh) was used

The Resin (30 g) was contained in a glass tube fitted with a sintered glass base (Quickfit and Quartz Ltd) The dimensions of the operational column were height/ 140 mm, diameter/15 mm

The column was washed with 250 ml 8 N hydrochloric acid and by 2000 ml water Immediately before use the column was washed with 100 ml N hydrochloric acid

Determination of the Alkaloid Content of Eluates.

The procedure of el DARAWY & TOMPSETT (1956) and TOMPSETT (1960 a) were used

A *Codeine, Brucine and Strychnine*

Before examination strongly acid eluates were neutralised by the addition of sodium bicarbonate

Before examination, an aliquot of eluates containing ethanol and ammonia were evaporated to dryness under reduced pressure The residue was dissolved in N hydrochloric acid Eluates containing ethanol and hydrochloric acid were treated similarly

B Morphine

The colorimetric method of Folin and Ciocalteu (TOMPSETT 1960 a) was employed Before examination, morphine was extracted from aqueous ammoniacal eluates with chloroform/isopropanol (TOMPSETT 1960 a)

The Elution of Alkaloids from the Cation Exchange Resin.

Under appropriate conditions, Dowex 50 × 12 will retain all substances possessing a basic group and these may be removed by hydrochloric acid of the appropriate normality (TOMPSETT 1960 b)

The eluting action of aqueous solutions of ammonia on these substances has been examined For this purpose the large column (described above and 300 ml of 6 N aqueous ammonia as eluant were used The results are shown in table 1 It would appear that only those

Table 1

The elution of basic substances absorbed on a column of Dowex 50 × 12 by aqueous ammonia (6 N)

Column weight 30 g
dimensions 140 × 15 mm

Eluted by Aqueous Ammonia	Not Eluted by Aqueous Ammonia
Glutamic acid	Choline
Glycine	Codeine
Cystine	Brucine
Arginine	Strychnine
Histidine	Hypotensive drugs –
Morphine	(a) Darenthin
p-Aminobenzoic acid	(b) Ismelin
p-Aminohippuric acid	(c) Hexamethonium
Sulphanilamide	
Nicotinic acid	
Nicotinamide	
p Aminophenol	
o-Tyrosine	
Tyramine	

Table 2

The recovery of alkaloids from a column of Dowex 50 W × 12 (30 g) by elution with ethanolic ammonia solutions

Alkaloid/Quantity	Nature of *Eluant*	Volume of *Eluant (ml)*	Recovery (%)
Codeine 5 mg	1	200	23 6
		400	43 4
	2	200	68 6
		400	89 6
	3	200	71 2
		400	96 3
Brucine 5 mg	1	200	56 6
		400	63 2
	2	200	63 2
		400	97 2
	3	200	64 2
		400	93 8
Strychnine 5 mg	1	200	13 4
		400	44 6
	2	200	72 0
		400	98 6
	3	200	90 6
		400	99 2
Composition of Eluant			
	Ammonia		4 N
	1 water/ethanol		(75/25)
	2 ditto		(50/50)
	3 ditto		(20/80)

substances possessing an acidic group e g phenolic, carboxyl, can be eluted by aqueous solutions of ammonia Morphine, because its phenolic group, can thus be readily separated from other alkaloids It was found that the purely basic substances, e g choline, brucine, strychnine and codeine, could be eluted from the column, after the ammonia treatment, by hydrochloric acid of the appropriate normality But 200 ml of ethanolic hydrochloric acid (90% ethanol/N hydrochloric acid) failed to remove any codeine, brucine or strychnine (5 mg) from columns

The alkaloids codeine, brucine and strychnine although not eluted from columns by aqueous ammoniacal solutions may be readily removed by treatment with solutions of ammonia in ethanol The results of such separation are shown in table 2 An alternative to elution by strong acids would sometimes be advantageous

The Examination of Tissues.

There appear to have been only two alternatives (Stewart *et al* 1937, Daubney & Nickolls 1937) described to the well established Stas – Otto

procedure The technique described below is suggested as another alternative, although its general application may be limited The procedure has been developed from that described for the separation of alkaloids from urine (TOMPSETT 1960)

A process for the separation of morphine from other alkaloids is included

A *Treatment of Tissue*

Liver tissue (100 g) was macerated in about 500 ml of water and 100 ml of 10 N hydrochloric acid when added The mixture was heated to boiling and then placed in a boiling water bath for 1 hour The mixture, after dilution to 1000 ml with water, was filtered and the small residue was washed with 500 ml of N hydrochloric acid

B *Ion Exchange Chromatography*

The filtrate was applied to a column The column was then washed with 500 ml of N hydrochloric acid and by 500 ml of water The column was then eluted with 300 ml of 6 N aqueous ammonia solution Morphine is recovered in this fraction The column was then washed with 200 ml water and 200 ml of N hydrochloric acid Codeine, brucine or strychnine were then eluted with 8 N hydrochloric acid

From the results shown in table 3, it will be seen that codeine, brucine,

Table 3

The recovery of alkaloids from a column of Dowex 50 × 12 (30 g) The alkaloid was applied as an acid extract prepared from 100 g of liver

Alkaloid	Quantity (mg)	Recovery of Alkaloid (%)
A *300ml of 6 N aqueous ammonia*		
Morphine	4	101
	8	95 5
	16	92 5
B *500 ml of 8 N aqueous hydrochloric acid*		
Codeine	4	81 6
	8	85 8
	16	92 9
Brucine	4	83 8
	8	82 5
	16	88 5
Strychnine	4	82 6
	8	83 8
	16	87 0

strychnine or morphine (4, 8 or 16 mg) added to 100 g of macerated liver could be recovered quantitatively by this technique

Summary.

Observations have been made on the use of a cation exchange resin to separate alkaloids from animal tissues as a preliminary to their identification and determination

A separation of morphine from other alkaloids and an alternative method of elution (codeine, brucine and strychnine) have been described

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